CLINICAL AND MICROBIOLOGICAL FEATURES OF PERIODONTAL DISEASE IN HIV-SEROPOSITIVE INDIVIDUALS

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Abstract

In addition to conventional periodontal disease Human Immunodeficiency Virus (HIV) seropositive patients can suffer from a range of periodontal diseases that are thought to result from immunosuppression. These include HIV-associated gingivitis, HIV-associated periodontitis and necrotising stomatitis. Furthermore, it has been suggested that periodontal tissue breakdown may be one of the first indications of HIV infection (Winkler and Murray, 1987). To determine the association between site-specific tissue breakdown and specific subgingival microorganisms a large number of sites need to be investigated. However, previous studies of the subgingival microbiota in HIV seropositive subjects have described data from only a few sites per subject. The development of DNA probes for the identification of subgingival microorganisms (French *et al*, 1986, Smith *et al*, 1988a, Gunaratnam *et al*, 1992) has allowed the rapid analysis of large number of subgingival plaque samples compared to traditional microbial identification techniques.

Twenty-nine HIV seropositive patients and 27 HIV seronegative patients were examined during the course of this study. Clinical data was collected from a total of 14,244 sites and subgingival plaque samples were collected from 1,461 sites. A total of 6,804 hybridisations were successfully completed identifying over 16,000 colonies to species level on nylon colony lifts.

On initial analysis, HIVseropositive subjects had a tendency for increased mean attachment loss compared to the HIV seronegative controls in this sample. The HIV seropositive subjects also harboured a higher mean percentage of *P. gingivalis,* and had a higher mean percentage of sites with suppuration than HIV seronegative subjects. A subgroup of nine HIV seropositive patients was identified with widespread periodontal disease, after criteria described by Haffajee *et al* (1992). These patients

differed in mean attachment loss, pattern of attachment loss, prevalence of clinical indicators and microbiology of subgingival plaque from the remaining 20 HIV seropositive patients with localised periodontal disease. Furthermore, these nine HIV seropositive patients with widespread periodontal disease were largely responsible for the differences observed between HIV seropositive and HIV seronegative groups as a whole. The incidence of HIV-associated periodontal diseases and the number of active sites found during the study was low.

In conclusion, this study confirms that there exists a subgroup of HIV seropositive patients who are at risk for an increased incidence of destructive periodontal disease. However, so many potentially confounding factors associated with HIV infection and treatment exist that identification of those patients who are at risk for periodontal disease may be difficult. Periodontal predictors of increased immunosuppression as HIV disease progresses are unlikely to be very powerful, even if they prove to be reliable, as not every patient with HIV infection in this study suffered from periodontal disease despite some patients having severe immunosuppression.

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List of Publications

The following papers and abstracts have been published as a result of research completed for this thesis.

1) Periodontal health and microbiology in HIV seropositive and HIV seronegative subjects

   D.L. Cross, G.L.F. Smith, D.H. Felix and D. Wray

   International Association for Dental Research, July 1992

   Journal of Dent. Res. 1992: 71: 606: Abs 722

2) Simultaneous hybridisation and colour detection of bacterial DNA on nylon colony lifts

   D.L. Cross, G.L.F. Smith and J.A. Ellender

   British Society for Dental Research, April 1993

   Journal of Dent. Res. 1993: 72: 4: Abs 191.

3) DNA probes in the detection of subgingival bacteria

   D.L. Cross

   Society for Anaerobic Microbiology.

   Biennial Meeting, Cambridge July 1993 (in press).

4) Simultaneous hybridisation and subsequent multicolour detection of subgingival bacterial DNA on colony lifts

   D.L. Cross, G.L.F. Smith, J.A. Ellender

   Archives of Oral Biology 1993 (in press).

Declaration

I declare that this thesis has been composed by myself and that the work described is my own.

Note on Taxonomy

Taxonomy of oral microorganisms is continually developing and a number of periodontal species have been recently reclassified under different genera. When referring to microorganisms used in this thesis the most recent names have been used. However, when referring to specific results, names as they appear in original research papers have been maintained .

List of Abbreviations

ACJ	amelocemental junction
AGE	agarose gel electrophoresis
AIDS	acquired immunodeficiency syndrome
AMLR	autologous mixed lymphocyte reaction
AMPPD	3-(2'-Spiroadamantan)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetan
ANUG	acute necrotising ulcerative gingivitis
ARC	AIDS-related complex
ATCC	American type culture collection
AVAP	AIDS virus-related periodontitis
BANA	benzoyl-DL-arginine-naphthylamide
BCIP	5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt
BMCMB	*Bacteroides* medium cooked meat broth
CDC	Centers for Disease Control
$CD4^+$	CD4 antigen positive (helper T-lymphocytes)
$CD8^+$	CD8 antigen positive (suppressor T-lymphocytes)
CFU	colony forming units
CMB	cooked meat broth
CTAB	hexadecyltrimethyl ammonium bromide
CTENP	mean attachment loss of worst 10% of sites in patient
dNTP	deoxyribonucleotide triphosphate
dATP	adenine triphosphate
dCTP	cytosine triphosphate
dGTP	guanine triphosphate
dTTP	thymine triphosphate
dUTP	uracil triphosphate

DMF	N,N-dimethylformamide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EDH	Edinburgh Dental Hospital
ELISA	enzyme linked immunosorbent assay
EOP	early onset periodontitis
FDC	Forsyth Dental Center
FITC	fluorescein isothiocyanate
GCF	gingival crevicular fluid
GDP	general dental practitioner
GJP	generalised juvenile periodontitis
HIV	human immunodeficiency virus
HIV -	HIV seronegative
HIV +	HIV seropositive
HIV-G	HIV-associated gingivitis
HIV-P	HIV-associated periodontitis
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin 1
IL-1α	interleukin 1-alpha
IL-1β	interleukin 1-beta
IL-2	interleukin 2
IL-6	interleukin 6
IVDA	intravenous drug abuser
kb	kilobase pairs
LJP	localised juvenile periodontitis
LPS	lipopolysaccharide
LTB4	leukotriene B4
NBT	nitroblue tetrazolium

NCTC	National collection of type cultures
NK	natural killer cells
NS	necrotising stomatitis
PCR	polymerase chain reaction
PCM	predominant cultivable microbiota technique
PGE_2	prostaglandin E_2
PGL	persistent generalised lymphadenopathy
PMNL	polymorphonuclear leucocytes
REA	restriction endonuclease analysis
RPP	rapidly progressive periodontitis
RNA	ribose nucleic acid
rRNA	ribosomal RNA
RTF	reduced transport fluid
SDS	sodium dodecyl sulphate
SE	standard error
SEM	scanning electron microscopy
SSC	saline sodium citrate
TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
TRITC	tetramethylrhodamine B isothiocyanate
TSBA	trypticase soy blood agar
% G+C	mole percent guanine plus cytosine content

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CHAPTER 1

LITERATURE REVIEW

1.1 CLINICAL DESCRIPTION OF PERIODONTAL DISEASE

In its broadest sense the term 'periodontal disease' may be used to describe any disease arising within or involving the periodontal tissues. Usually, however, the term is used to describe a range of clinical conditions that essentially involve two processes i.e. gingivitis and periodontitis. Both conditions can exist concurrently at the same site, or individually at different sites within the same mouth and are generally agreed to be the result of host-bacterial interactions. The distinction between the two conditions depends on whether apical migration of the periodontal attachment from the ACJ has occurred, the definition of periodontitis. Although traditionally gingivitis and periodontitis have been divided into acute and chronic conditions, in the majority of cases they are chronic conditions, which can persist for many years (Williams *et al*, 1992).

1.1.1 Gingivitis

Clinically normal healthy gingiva has a pink appearance, firm consistency and a scalloped gingival margin. The interdental papillae are firm, completely fill the interdental space and do not bleed on gentle probing. (Lindhe, 1989). Histological signs of inflammatory changes in gingival tissue develop within approximately 48 hours of plaque accumulation and after 10 to 20 days, overt signs of gingival inflammation can be observed clinically in most individuals in the continued absence of oral hygiene. If microbial plaque is removed and adequate oral hygiene procedures instigated, then these inflammatory changes are reversed and a clinically healthy appearance can be restored after a few days (Löe *et al*, 1965; Theilade *et al*, 1966).

1.1.2 Classification of Gingivitis

1.1.2.1 Chronic or adult gingivitis

Chronic gingivitis can be defined as inflammation of the marginal gingival tissues due to the accumulation of dental plaque and is characterised clinically by redness, swelling, and increased tendency to bleed on probing. Histologically, inflammatory changes do not involve the underlying periodontal ligament and alveolar bone. The epithelial attachment does not extend apically beyond its normal position at the amelocemental junction. Chronic gingivitis is almost universally prevalent in the adult population, however there is wide variation in the clinical signs of gingivitis between subjects. Although the most obvious differences between individuals can be ascribed to levels of dental plaque, either generally or specifically associated with local plaque traps, different subjects appear to have different sensitivities to the effects of a given quantity of dental plaque. Chronic gingitivis can remain unchanged for many years or may result in the development of periodontitis.

1.1.2.2 Acute necrotising ulcerative gingivitis

ANUG mainly affects young adults with poor oral hygiene in conjunction with other predisposing factors such as smoking, concurrent viral infections and a defective immune response (Johnson and Engel, 1985; Williams *et al,* 1992). It is a destructive inflammatory gingival condition characterised clinically by interproximal necrotic ulcers in addition to those features described for chronic gingivitis. These lesions are extremely painful and are often covered by a slough or pseudomembrane. Affected sites commonly have a pre-existing chronic gingivitis. Other features can include, linear erythema, bleeding, foetor oris, lymphadenitis, fever and malaise. (Lindhe, 1989). If

untreated these ulcers may enlarge and spread rapidly to involve the marginal gingivae, and occasionally the attached gingivae. The condition may resolve after a few days, but is prone to recur. Alternatively, the acute phase of the disease can subside and chronic necrotising gingivitis can ensue, which continues to destroy the periodontal tissues but at a slower, less dramatic rate.

1.1.2.3 Childhood gingivitis

Although gingivitis is very rare before the age of six years, its prevalence increases gradually until puberty when over 90% of children are affected These lesions are reported to have a predominately T-lymphocyte infiltrate (Seymour *et al*, 1981; Williams *et al*, 1992).

1.1.3 Periodontitis

Chronic periodontitis can be defined as plaque induced inflammation of the periodontal tissues which results in the destruction and loss of the periodontal ligament and crestal alveolar bone with concomitant apical migration of the epithelial attachment (Williams *et al*, 1992). Over time this can result in the formation of a periodontal pocket produced by the continued migration of the junctional epithelium on to the root surface. Ultimately, the destruction of the supporting tissues may result in exfoliation of teeth. This process is the major cause of tooth loss in adults (Listgarten, 1986a,b).

1.1.4 Classification of Periodontitis

1.1.4.1 Adult periodontitis

Adult periodontitis is the most common form of periodontitis and is characterised by inflammation and loss of periodontal attachment which usually starts after 30 years of age. There is considerable variation in disease expression between subjects suffering from disease classified as adult periodontitis. Patients with adult periodontitis that does not respond to normal treatment procedures have been grouped into an additional category called refractory periodontitis (Lindhe and Nyman, 1984; Adams, 1992).

1.1.4.2 Early onset periodontitis

Early onset periodontitis includes a number of clinical conditions that affect a small percentage of young people who appear to be especially susceptible to periodontal disease characterised by rapid progression.

1.1.4.2.1 Localised juvenile periodontitis

LJP is characterised by severe rapid periodontal disease of the permanent first molars and incisor teeth with onset around puberty. Typically, it is not associated with gross plaque deposits and the affected tissues show little inflammation clinically. The recovery of *Actinobacillus actinomycetemcomitans* from a high proportion of affected sites from patients with LJP has implied a specific aetiological role for this organism in the disease process (Slots *et al*, 1980; Zambon *et al*, 1983a,b). Generalised juvenile periodontitis is described as clinically more extensive than LJP, affecting several teeth in other segments in addition to the first permanent molars and incisors. There is debate at present regarding LJP and GJP. Some workers believe that LJP and GJP are separate

disease entities, others that GJP represents a more aggressive form of LJP or is the result of untreated LJP (Lindhe, 1989).

1.1.4.2.2 Rapidly progressive periodontitis

This subset was proposed in the light of longitudinal studies indicating that approximately 10% of adult periodontitis patients developed a more rapidly progressing form of the disease (Hirschfield and Wasserman, 1978; McFall, 1982; Page *et al*, 1983a) The age of onset tends to be after 20 years and the disease is characterised by generalised severe periodontal destruction. During active phases the gingival tissues are described as being very inflamed, exhibiting haemorrhage, exudation of pus and proliferation of the marginal gingivae. Soft tissue destruction and alveolar bone loss can occur very rapidly and may be accompanied by systemic signs i.e. general malaise, weight loss and depression. Furthermore, defects in either neutrophil or monocyte chemotaxis have been reported in some of these patients (Page *et al*, 1983a).

1.1.4.2.3 Prepubertal periodontitis

Destructive periodontal disease of the primary dentition is extremely rare, although localised and generalised forms have been described (Page *et al*, 1983b). Generalised prepubertal periodontitis is associated with severe gingival inflammation, gingival tissue proliferation, gingival recession and widespread, rapid destruction of alveolar bone. It is a rapidly progressive disorder that can lead to early loss of the deciduous dentition and may involve permanent teeth. Localised prepubertal periodontitis only affects the periodontal tissues at one or more primary molars and is associated with moderate clinical signs of inflammation, deep periodontal pocketing and localised bone loss are features of affected sites. (Page *et al*, 1983b; Lindhe, 1989).

1.2 DENTAL PLAQUE

Dental plaque has been defined as the bacterial aggregations that form on the teeth and other solid oral structures (Lindhe, 1989). It consists of microbial cells and their products together with host cells and compounds mainly derived from saliva (MacFarlane and Samaranayake, 1989). Dental plaque present in the dentogingival area can be classified as supragingival, being deposited on the clinical crowns of teeth, and subgingival, located in the gingival sulcus or periodontal pocket (Lindhe, 1989). Supragingival and subgingival plaque have different physical appearances that reflect differences in the supragingival and subgingival environments and microbiota.

1.2.1 Supragingival Plaque

1.2.1.1 Early plaque formation

Plaque begins to re-establish supragingivally within a few minutes of cleaning teeth with the formation of an organic film or pellicle on exposed tooth surfaces (Van Houte *et al*, 1971). The acquired pellicle is produced by the reaction of glycoproteins, lysozyme and immunoglobulins present in saliva with the clean hydroxyapatite surface (Williams *et al*, 1992). After cleaning, bacterial recolonisation of the tooth surface is achieved by organisms adhering to the pellicle from saliva and organisms that remained in less accessible areas of the tooth surface. Early colonisers of the tooth surface are mainly Gram-positive cocci and rods, for example *Streptococcus sanguis*, *Actinomyces viscosus* and *Actinomyces israelii*. Early bacterial plaque contains few, if any, Gram-negative species (Gibbons and van Houte, 1973).

1.2.1.2 Plaque development

In a classic experimental model of gingivitis, Löe *et al* (1965) and Theilade *et al* (1966) described three distinct phases of plaque formation in human volunteers. Following professional plaque removal the volunteers abstained from normal oral hygiene procedures for up to three weeks. The clinical and microbiological changes in Gram stain and morphology of plaque bacteria were noted. The first phase, observed from day zero to day two, was associated with a proliferation of Gram-positive cocci and rods, with Gram-negative cocci and rods contributing up to 30% of species. These proportions are similar to those found in whole saliva. The second phase, observed from day one to day four, exhibited an appearance and increase of *Fusobacteria* and filaments. Spirochaetes appeared during the third phase, observed from day four to day nine, and were associated with clinically detectable gingivitis.

Cultural studies have revealed that an orderly succession of species is observed as supragingival plaque matures. Initially, *Streptococci* and *Actinomyces* dominate but during the next three weeks of plaque accumulation *Fusobacterium*, *Veillonella*, *Treponema* and *Bacteroides* species appear (Socransky *et al*, 1977a,b; Syed and Loesche, 1978). The microbiological development of supragingival plaque is believed to be due to ecological changes in the local environment related to bacterial interactions and the availability of nutrients (Socransky, 1977b).

Supragingival plaque organisms utilise dietary carbohydrates and salivary components as their main source of nutrients (Williams *et al*, 1992). Several species produce an extracellular polysaccharide matrix that can contribute up to 50% of total plaque bulk (Lindhe, 1989). This matrix contributes to the semi-solid, adherent nature of supragingival plaque.

1.2.2 Subgingival Plaque

Initially, subgingival plaque develops as an extension of supragingival plaque and is believed to become established as early as four weeks after the onset of clinical gingivitis (Williams *et al*, 1992). Initial colonisers are Gram-negative cocci, rods and *spirochaetes*, however a diverse flora quickly develops that reflects different ecological pressures present in the subgingival environment (Lindhe, 1989). Potential colonisers of the subgingival area have to utilise nutrients that are derived from gingival crevicular fluid as opposed to saliva, cope with a lower oxygen concentration and negotiate host defence mechanisms (Williams *et al*, 1992). As subgingival plaque matures the microbiota consists predominantly of obligate anaerobes or facultative anaerobes, many of which are motile including *Porphyromonas*, *Fusobacterium*, *Selenomonas*, *Capnocytophaga*, *Eikenella*, and *Vibrio* species. These organisms do not produce extracellular polysaccharide matrix for support, but depend instead on interbacterial adherence and attachment to the host (Williams *et al*, 1992). Subgingival plaque is therefore generally less dense than supragingival plaque, although it can have both solid and semi-liquid states (Christersson *et al*, 1991).

Over time the subgingival microbiota becomes more complex and over 300 species have been isolated from subgingival plaque (Moore *et al*, 1985). In advanced periodontal pockets the cultivable flora is dominated by anaerobic species, the majority of which are Gram-negative rods including, *Bacteroides* spp., *Fusobacterium nucleatum* (Tanner *et al*, 1984) and *spirochaetes* (MacPhee and Muir, 1986).

1.3 HISTOLOGY

1.3.1 Periodontal Disease

Histological and electron microscopic studies of gingivitis and periodontitis provide valuable information about the cellular events that occur during these disease processes and permit speculation about the pathogenic mechanisms that are responsible for the observed tissue changes. Although the histological and ultrastructural features have been divided into stages, the disease process represents a continuum and these stages do not distinguish the sequence of signs and symptoms observed clinically.

Page and Schroeder (1976) summarised the histological and ultrastructural events observed in the development of human and animal gingivitis and periodontitis. Their work indicates a sequence of four stages in the pathogenesis of periodontal disease beginning with the initial accumulation of supragingival plaque at previously healthy sites.

1.3.1.1 Initial lesion

The initial lesion develops within two to four days of plaque accumulation and is confined to the region of the gingival sulcus. During this stage vessels of the gingival plexus become engorged and dilated, and large numbers of PMNL migrate into the junctional epithelium and gingival sulcus. A few macrophages and blast-transforming lymphocytes may appear in the connective tissue and junctional epithelium. Perivascular collagen is lost and is replaced by fluid, serum proteins and inflammatory cells. These changes are characteristic of an acute inflammatory response and are believed to be the result of chemotactic and antigenic substances in the gingival sulcus region.

1.3.1.2 Early lesion

The early lesion evolves from the initial lesion within four to seven days after the beginning of plaque accumulation although there is no clear dividing line between the two. As the growth of plaque continues, the features described above become more marked and the inflammatory infiltrate becomes more extensive. There is an increase in the number of macrophages and lymphocytes present, with initially both T and B cells present, although T cells dominate towards the end of the second week of plaque accumulation. In the early stages plasma cells are normally present only at the lateral and apical periphery of the infiltrate. Fibroblasts begin to show signs of cell damage including enlargement, swollen mitochondria, vacuolisation of the rough endoplasmic reticulum and rupture of the cell membrane. Marked collagen loss is evident near the junctional epithelium and may reach 60 - 70%. Rete peg proliferation can be observed in the coronal part of the junctional epithelium.

The features of chronic inflammation begin to dominate the lesion as time progresses, however the features of an acute inflammatory response, including crevicular fluid flow and PMNL migrating into the gingival crevice persist.

1.3.1.3 Established lesion

The established lesion develops within two to three weeks of plaque accumulation and is characterised by a predominance of plasma cells within the inflammatory infiltrate. The extent of the inflammatory infiltrate has increased both laterally and apically, although it is still centered around the base of the gingival sulcus and junctional epithelium. These plasma cells are actively engaged in the production of immunoglobulins which are released locally and pass through the junctional epithelium to enter the gingival crevice. Plasma cells are no longer confined to the reaction site but

may also be found along blood vessels and between collagen bundles deep within the connective tissues. The migration of PMNL into the gingival crevice becomes more marked and as a consequence the junctional epithelium is heavily infiltrated with these cells. Once in the gingival crevice they attach to the outer surface of the plaque to form a layer between it and the junctional epithelium. The coronal portion of the junctional epithelium and oral sulcular epithelium can show marked lateral proliferation into the connective tissue although there is as yet no apical migration of the epithelial attachment. The junctional epithelium may be thinned and detached from the tooth surface in places and ulceration can occur. Continuing loss of collagen is evident throughout the connective tissue in the zone of infiltration and in more distant regions, however loss of alveolar bone or connective tissue attachment are not seen. In addition to those described, features of the earlier stages of the lesion are also present, often in an accentuated form. This stage represents a stable interaction between dental plaque and host defences that may persist for months or even years without any signs of further disease progression.

1.3.1.4 Advanced lesion

The advanced lesion as described by Page and Schroeder (1976) represents destructive periodontitis and includes loss of alveolar bone and periodontal ligament, fibrosis of the gingiva and pocket formation.

The lesion is no longer localised and features of the acute inflammatory response persist in the presence of a chronic inflammatory infiltrate extending apically as well as laterally. In addition, a dense infiltrate of plasma cells, lymphocytes and macrophages are observed. At its deepest extent it is often focal and perivascular, separated by bands of fibrous tissue. Lateral proliferation and ulceration of the junctional epithelium continue to be a feature along with apical migration of the epithelial attachment. This

event coincides with pocket formation and permits the extension of subgingival plaque on to the root surface. Bone destruction begins along the crest of the interdental septum around communicating blood vessels some distance from the inflammatory infiltrate, often separated from it by trans-septal collagen fibres. Although these fibres appear to be continuously regenerated as the lesion progresses apically, other collagen fibre bundles loose their orientation and architecture completely. Collagen fibres are practically absent within the hypercellular infiltrated connective tissue, whereas dense fibrosis is evident in the surrounding area.

The advanced lesion can, like the established lesion, become stable. Active periodontal destruction is believed to occur in an episodic manner, interspersed with period of quiescence. This is discussed in section 1.7.

1.3.2 Plaque

1.3.2.1 Supragingival

The gross histological features of supragingival plaque were discussed in the previous section.

1.3.2.2 Subgingival

Ultrastructural studies have revealed that subgingival plaques comprise a variety of bacterial morphotypes and exhibit considerable variation between specimens (Listgarten, 1976; Newman, 1976). In a recent study of patients with chronic periodontitis, Vrahopoulos *et al* (1992) reported that established subgingival plaque consisted of three to four layers, from the root surface to pocket epithelium. The first layer nearest the cementum consisted of densely packed Gram-positive coccoid cells

arranged perpendicular to the root surface in palisades. Although Gram-negative cocci and rods were predominant in the middle layers, Gram-positive filaments were also common. Spirochaetes were usually close to the superficial layer, randomly distributed among the other forms. The most superficial layer exhibited three general patterns depending on the density of bacterial cell packing. The loose type of superficial layer was the most common and comprised a variety of bacterial forms usually without orientation, including spirochaetes, Gram-negative filaments, coccoid and rod-shaped cells. Corn-cob, rosette and tooth brush forms were observed at the outermost level of this layer. The dense type of superficial layer was restricted to Gram-negative and Gram variable coccoid and rod-shaped bacteria with a few filaments and *spirochaetes*. A layer of erythrocytes was frequently found at the pocket surface of these types of plaques. The very dense type of superficial layer consisted of closely packed bacteria, predominantly Gram-negative and Gram variable cocci, short rods and filaments, with the occasional spirochaete. A feature of these plaques was a layer of PMNL lining the pocket surface. Apically, subgingival plaque has been found to vary in its termination ranging from a long gradual reduction in bacteria, to a series of bacterial islands of Gram-negative cocci and short rods with occasional spirochaetes extending into a plaque free zone (Vrahopoulos *et al*, 1992). In a recent SEM study of extracted healthy and periodontitis-affected teeth, Friedman *et al* (1992) reported that microorganisms were always present in the "plaque-free zone" and that in healthy specimens the apical border plaque consisted mainly of cocci and short rods whereas in diseased teeth spirochaetes predominated.

1.4 ECOLOGY OF PERIODONTAL POCKET

1.4.1 Acquisition and Development of the Oral Microbiota

During the first few months of life an infant relies on passive immunity, acquired from its mother, for protection against infection. Passive immunity is believed to exert a strong influence on the composition of the resident microbiota that colonises the skin and mucous membranes of the infant (Lehner, 1982). In turn, the acquired microbiota is thought to influence some of the characteristics of the developing immune system. This interplay between immune system and microbiota continues throughout the life of the individual and results in the establishment of a commensal microbiota of skin and mucous membranes that is a prerequisite for health (Listgarten, 1986a). There is believed to be an element of chance involved in which organisms initially colonise a particular site and thus fill the related niches in the ecosystem. These initial random events thereby influence the sequence of subsequent ecological succession (Donoghue, 1990; Socransky *et al*, 1988a).

Using predominant cultivable microbiota techniques, Könönen *et al* (1992 a,b), investigated the colonisation by several Gram-negative anaerobic bacteria in 30 edentulous infants and their mothers. These workers found that various bacterial species readily colonise the oral cavity of edentulous infants, including *Prevotella intermedia, Fusobacterium nucleatum, Eikonella corrodens, Prevotella melaninogenicus, Bacteriodes gracilis* as well as *Veillonella, Wolinella, Capnocytophaga* species and non-pigmenting *Prevotella* species. They also found that the number of different anaerobic species in the same infant mouth increased in direct proportion to age. They concluded that maternal saliva could act as a source of some Gram-negative anaerobes and that these could become established in the oral microbiota of edentulous infants (Könönen *et al*, 1992b).

It has been known for some time that the newly formed gingival crevice is quickly colonised, following the eruption of teeth, by organisms frequently isolated from the oral cavity of adults (Gibbons and van Houte, 1975). However, *A. actinomycetemcomitans* and *Porphyromanas gingivalis* are rarely detected in edentulous infants (Könönen *et al*, 1992a), young children (Fisken *et al*, 1990) or prepubertal children (Van Oosten, 1988). This observation has increased speculation that *A. actinomycetemcomitans* and *P. gingivalis* may be exogenous pathogens in some cases of destructive periodontal disease (Genco *et al*, 1988; van Oosten *et al*, 1988). The hormonal events of puberty are believed to cause further shifts in the oral microbiota. Van Oosten *et al* (1988) supported the theory that *Bacteroides melaninogenicus* and *Bacteroides intermedius* were commensal organisms that were given the opportunity to increase during puberty as a result of the hormonal imbalance at this time.

Throughout the remainder of life, in addition to providing nutrients such as hemin, long-chain fatty acids and serum proteins via the GCF, the immunologically competent host is believed to exert ecological pressures on the periodontal pocket via non-specific and specific defence mechanisms (Theilade, 1986). Non-specific mechanisms include desquamation of pocket epithelial cells, PMNL, lysozyme and other bacteriocidal enzymes and products in GCF. Specific defence mechanisms include the production of specific antibodies that may aid phagocytosis of selected organisms or prevent them from colonising the periodontal pocket. There is some evidence to suggest that the composition of the oral microbiota and the incidence of some periodontal species continue to develop throughout the life of the individual in response to these changes (Savitt and Kent, 1991; Könönen *et al*, 1991).

A variety of mechanisms have been attributed to several periodontal bacteria that allow them to colonise, evade host defences and cause tissue damage. These attributes have been called virulence factors and will be discussed in section 1.6.4.1

1.4.2 Bacterial Interactions

The existence of an ordered structure to subgingival plaque (sections 1.2 and 1.3), including the presence of formations such as palisading Gram-positive cocci, corn cob, rosette and toothbrush formations (Listgarten, 1976; Vrahopoulos *et al*, 1992), supports the evidence that subgingival plaque contains a complex community of organisms that are involved in commensal and antagonistic relationships. These range from physical to nutritional and biochemical synergistic and antagonistic relationships (Grenier and Mayrand, 1986; Ter Steeg and Van Der Hoeven 1989; Ohta *et al*, 1991; Hillman and Socransky, 1982; Hillman *et al*, 1985). Some bacterial interactions could be interpreted as potentially detrimental to the host by promoting the colonisation and growth of suspected periodontal pathogens (Socransky *et al*, 1988b; Socransky and Haffajee, 1991; Socransky and Haffajee, 1992). However, others may be of benefit to the host. For example, a species may occupy a niche which would otherwise be colonised by a pathogen, or may actively inhibit its growth. Alternatively, a species may inhibit the action of or degrade virulence factors produced by a pathogen that would otherwise have caused tissue damage (Socransky and Haffajee, 1992). One example of interbacterial antagonism occurring between periodontal bacteria involves *A. actinomycetemcomitans* and the following species, *Streptococcus sanguis, Streptococcus uberis* and *Actimomyces viscosus*. Hillman *et al*, (1985) showed that the latter species produced hydrogen peroxide, either directly or via a host peroxidase system that inhibited *A. actinomycetemcomitans*. Similarly, *A. actinomycetemcomitans* was shown to produce a bacteriocin that specifically inhibited growth of *S. sanguis,*

S. uberis and *A. viscosus* species, but not others (Hammond *et al*, 1987; Stevens *et al*, 1987) The effects of this mutual antagonism were observed by Socransky *et al* (1988a) who reported decreasing odds of detecting *A. actinomycetemcomitans* across sites with increasing levels of *S. sanguis*.

However, the oral cavity is an open ecosystem, and as such is continually exposed to new microbial species and potential colonisers (Midtvedt, 1990). This means that in addition to indigenous species comprising the normal resident flora, a transient flora can also develop in a niche from time to time that comprises organisms "just passing through" or temporarily filling a niche vacated by its normal resident. These organisms are generally not ideally suited to the environment and usually disappear (Midtvedt, 1990). Therefore resident microbiota of the subgingival crevice is thought to be of crucial importance in the defence against colonisation by possible periodontal pathogens and potential extraneous colonisers (Preus, 1990). The property of resident species to exclude potential extraneous colonisers can be termed colonisation resistance and arises after the microbial population has become established, and inter-relationships have developed, to produce a synergistically integrated microbial flora (Donoghue, 1990). The effectiveness of colonisation resistance has been demonstrated in caries research, with the ability of some dental plaques to resist colonisation by *S. mutans* (Perrons and Donoghue, 1990).

As indicated above, the host plays an integral part in the development and maintenance of the microbial environment and this is reflected in the observation that immunocompromised hosts are prone to overgrowth of minor components of the flora or colonisation by extraneous organisms (Samaranayake *et al*, 1984; Dreizen *et al*, 1986). Colonisation resistance may also be reduced in resident microbiota by the effects of systemic broad-spectrum antimicrobials, the consequences of which have been well documented (Gill and Pallach, 1981; Gordon *et al*, 1985). Indeed, Slots *et al* (1988,

1990) and Rams *et al* (1992) concluded that the high percentage of *Enterobacteraceae*, yeasts and *Pseudomonas* spp found in refractory sites from severe periodontitis patients may be the result of prior systemic antimicrobial therapy. A recent study by Helovuo *et al* (1993) reported that 42% of chronic periodontitis patients that had been prescribed broad-spectrum antimicrobials, for the treatment of other diseases, developed a purulent form of periodontal superinfection. These authors concluded that broad-spectrum antimicrobials, in the absence of subgingival debridement and professional cleaning could lead to a dramatic increase in the prevalence of enteric rods, staphylococci and yeasts in the subgingival microbiota.

In summary, the oral cavity is initially colonised, at different sites, by a mixture of various species largely by chance. Given time, the resident flora at a given periodontal site develops, through host and bacterial inter-relationships, into a complex microbial ecosystem. In health, the resident microbial flora in a subgingival crevice would be expected to have the property of colonisation resistance and inhibit or prevent colonisation by new or extraneous species.

The relationship between ecology and periodontal disease is discussed further in section 1.6.3.3

1.5 MICROBIAL IDENTIFICATION TECHNIQUES

1.5.1 Cultivation

Traditional methods of identification of organisms associated with periodontal diseases have relied on culture of subgingival plaque samples. For example, the predominant cultivable technique has been used in numerous studies at a limited number of centres to characterise the predominant cultivable microflora associated with healthy sites (Slots,

1977a), adult periodontitis sites (Slots, 1977b; Dzink *et al*, 1985, 1988; Moore *et al*, 1991), and juvenile periodontitis sites (Newman and Socransky, 1977; Haffajee *et al*, 1984; Moore *et al*, 1985). This technique involves the preparation of primary isolation cultures of dispersed and diluted plaque samples, on non-selective media. After anaerobic incubation, 30 - 50 colonies are chosen at random, subcultured to purity and identified by Gram stain, biochemical tests and end product analysis e.g. gas liquid chromatography.

In the early 1980's, existing concepts of disease activity were challenged and it was proposed that periodontal destruction was not continuous and uniformly distributed as once thought, but episodic and site-specific (Goodson *et al*, 1982; Socransky *et al*, 1984). The results of PCM studies of active and inactive periodontitis sites (Dzink *et al*, 1985, 1988; Haffajee *et al*, 1988a,b), led to speculation that only a limited number of species from several hundred that had been isolated from subgingival pockets (Moore *et al*, 1985) were associated with episodes of periodontal disease activity. Furthermore, it appeared that some species were beneficial to the health of the periodontium.

Although, the PCM provides a very wide picture of the subgingival microbiota, there are several disadvantages to this approach that make it unsuitable for the detection of a limited number of species in a large number of plaque samples. Subculture and identification of randomly selected colonies on a primary isolation culture is time-consuming, labour-intensive and depends ultimately on the viability of organisms in the sample. For these reasons a number of rapid microbial identification methods have been developed and applied to the search for suspected pathogenic and beneficial species in subgingival plaque samples.

1.5.2 Immunological Techniques

1.5.2.1 Immunofluorescence microscopy

Immunofluorescence microscopy involves the use of either monoclonal or polyclonal antibodies raised against specific periodontal species (Zambon *et al*, 1985, 1986; Gmür, 1988). Typically, a subgingival plaque sample is spread on to a glass slide and fixed before a species-specific or serotypically specific, monoclonal or polyclonal antibody is added. This antibody can either be conjugated to a fluorescent substrate e.g. FITC or TRITC and fluorescence viewed directly, or it can be detected indirectly via intermediate substrates which allow amplification of the signal before the fluorescent substrate is added. A microscope with appropriate filters is used to view random fields of plaque sample and count the number of cells exhibiting fluorescence. This count is then compared to a total count of the same field viewed under phase contrast. Using this approach Zambon *et al*, (1985), reported sensitivity of between 91 to 100% and specificity of between 87% to 89%.

This approach has the advantage that the plaque sample is viewed directly and does not rely on culture. However there are a number of disadvantages. Cross-reactivity of monoclonal and polyclonal antibodies with several subgingival species appears to be a common occurrence (Mouton *et al*, 1980; Gmür and Guggenheim, 1983) and strict testing of the specificity of the antibodies is advocated (Gmür, 1988). The production of both monoclonal and polyclonal antibodies is time-consuming and technically demanding. Whole non-viable target cells can be included in the total count as they continue to exhibit surface antigens after death. There is also concern that host-derived antibodies to the same bacterial epitopes targeted by monoclonal and polyclonal antibodies may cause masking and therefore lead to false negative results (Gmür and Guggenheim, 1990). Finally, microscopic examination of many plaque samples can be

tedious, and require concentration and experience, factors which could limit the number of samples assayed at one time (Gmür, 1988).

1.5.2.2 Bacterial concentration fluorescence immunoassay

Wolff *et al* (1992) reported the development of a fluorescence immunoassay that is performed in specially designed microtitre plates. The base of the wells in the microtitre plate is a membrane filter that is situated above a waste reservoir. The washed and resuspended plaque sample is added to a well along with species-specific FITC-labelled monoclonal antibody. After a short incubation, the well is washed and the contents filtered through the base of the well into the waste reservoir under vacuum. This removes unbound monoclonal antibody and allows the total bacterial bound fluorescence to be determined by a fluorimeter. The relative bacterial cell equivalents are determined by comparison to standard curves. This technique has the advantage of being relatively fast. However many of the disadvantages of the use of immunofluorescence microscopy noted above still apply. Sensitivity levels were reported to be between 97% to 100%, and specificity ranged between 57% to 92% (Wolff *et al*, 1992).

1.5.2.3 Chairside polyclonal assay - "Evalusite"

Polyclonal antibodies to specific suspected periodontal pathogens have been developed by Eastman-Kodak into a chairside assay for the presence of *P. intermedia, P. gingivalis* and *A. actinomycetemcomitans*. This system is based on a sandwich enzyme immunoassay approach (Contestable *et al*, 1990). Discs of a porous membrane are impregnated with polyclonal antibodies specific to the three organisms in three distinct areas. A subgingival plaque sample is collected by sterile paper point and deposited into an eluting solution. This is then added to one of the test discs and left to

incubate at room temperature for a few minutes. During this time organisms expressing these specific antigens are bound to the disc at the corresponding antibody dot site. A second antibody, conjugated to horseradish peroxidase, is then added that binds to any trapped antigen. Finally a colour development reagent is added that produces a precipitate in the presence of the enzyme. The intensity of the colour precipitate at each of the three sites is compared to a colour card supplied with the kit. The sensitivity of this system is difficult to determine although the manufacturers claim a 90% specificity (Contestable *et al*, 1990).

1.5.3 Restriction Enzyme Analysis

Restriction endonucleases can recognise and cleave double stranded DNA at the site of specific base-pair sequences. The resulting fragments can be resolved by agarose gel electrophoresis, ethidium bromide staining and visualised under ultra violet light (Voytas, 1989). The number and size of DNA fragments obtained from a bacterial strain constitute a specific fingerprint, which can be compared against other strains. REA is especially useful in characterising the genetic heterogeneity within a species (Loos *et al*, 1990; Chen *et al*, 1990; Han *et al*, 1991) and in epidemiologic studies to investigate source and mode of transmission (Zambon *et al*, 1990; Genco and Loos, 1991). Furthermore, REA may be used to construct cloned DNA probes (see below).

1.5.4 Nucleic Acid Probes

Whole chromosomal (Savitt *et al*, 1988; Albandar *et al*, 1990; Lippke *et al*, 1991; Gunaratnam *et al*, 1992; Haffajee *et al*, 1992), cloned (French *et al*, 1986; Savitt *et al*, 1988; Albandar *et al*, 1990; Zappa *et al*, 1990; DiRienzo and Slots, 1990) and oligonucleotide (Dix *et al*, 1990; Maiden *et al*, 1992) nucleic acid probes have been used for the detection of periodontal species in subgingival plaque samples. Isotopic

and non-isotopic labels have been used in conjunction with the various probe types listed above.

Whole chromosomal DNA probes are constructed by simply labelling whole chromosomal DNA extracted from pure culture of the species under investigation. However, there are potential problems with cross-reactions with genetically similar organisms under conditions of low stringency (Savitt *et al*, 1990; Dix *et al*, 1990). Whole chromosomal DNA probes have a lower limit of detection of 10^3 - 10^4 organisms (Savitt *et al*, 1990; Gunaratnam *et al*, 1992) with sensitivities and specificities in the range 98% to 100% under strictly controlled conditions of high stringency (Gunaratnam *et al*, 1992).

Cloned probes are produced by first isolating a specific DNA segment by restriction endonuclease analysis and gel electrophoresis. Specific fragments are recovered, integrated into a vector, e.g. plasmid DNA, and used to infect a rapidly multiplying and easily cultured species such as *E. coli*. Multiple copies of the specific sequence are produced which are then purified and labelled (French *et al*, 1986; DiRienzo and Slots, 1990; DiRienzo *et al*, 1990; Bolstad *et al*, 1991; DiRienzo *et al*, 1991). The sensitivity of cloned DNA probes is comparable to that reported for whole chromosomal probes, but they have the advantage that they can be species-specific and cross-reactions between closely related species are avoided (French *et al*, 1986; Savitt *et al*, 1988).

Oligonucleotide DNA probes are typically 10 - 40 bases long and are synthesised base by base to match species-specific sequences of nucleic acid, either in chromosomal DNA or ribosomal RNA. These probes can have very short hybridisation times compared to other probe types and are highly specific, however a disadvantage of their size is that only one reporter molecule can be bound to each probe. If an oligonucleotide probe has been synthesised to target regions of chromosomal DNA, it

may not be as sensitive as whole chromosomal or cloned probes (Albandar and Olsen, 1990). This is due to the number of copies of target DNA sequence per cell and the effect of probe size on the number of reporter molecules bound to each probe, although strategies have been developed that can enhance the sensitivity of oligonucleotide probes (Tenover, 1988).

Species-specific oligodeoxynucleotide probes synthesised to target regions of 16S rRNA for periodontal organisms have been described (Chuba *et al*, 1988; Dix *et al*, 1990). This approach has the advantage that ribosomal RNA is present in cells in higher amounts than DNA and so there are multiple copies of the target sequence contained in each cell. Dix *et al* (1990) reported good specificity with sensitivity in the order of 10^3 cells for oligodeoxynucleotide probes directed against a range of ten periodontal species. However, rRNA content of cells can vary enormously and make quantitation of signals difficult (Savitt *et al*, 1990).

1.5.5 Polymerase Chain Reaction

Identification of different strains can also be achieved using products from the PCR technique. Griffen *et al* (1992) described a technique that amplifies the highly variable region between the 16S and 23S ribosomal genes by PCR and uses REA on the products to differentiate between strains. This technique is claimed to be more precise and reproducible than REA of whole chromosomal DNA noted above. Studies of 16S rRNA genes retrieved directly from plaque samples using PCR products have recently been reported (Wilson, 1993). The PCR technique can also be used to type bacteria by amplifying whole chromosomal DNA with random primers and resulting products compared by AGE (MacGowan *et al*, 1993).

1.5.6 Enzymatic

Loesche *et al* (1987, 1990a) described the development of an enzymatic test to detect *T. denticola, P. gingivalis* and *B. forsythus* in subgingival plaque samples. These organisms can hydrolyse BANA (benzoyl-DL-arginine-naphthylamide), a synthetic trypsin substrate. Decreased numbers of BANA positive sites have been demonstrated following successful treatment of periodontal lesions, while clinical deterioration and recolonisation of sites were associated with an increase in BANA positive sites (Bretz *et al*, 1991). Sensitivity and specificity of the test have been reported to be 87% and 73% respectively (Loesche *et al*, 1990b). Although this technique has the advantage of being rapid and assays the plaque sample directly, it cannot differentiate between these three species and is therefore nonspecific. A commercial chairside kit for the rapid detection of these three species in subgingival plaque samples has been developed utilising BANA hydrolysis and is marketed under the name of "Perioscan".

1.5.7 Comparative Studies

A number of studies have compared the relative sensitivity and specificity of these rapid methods of identification of organisms with traditional cultural techniques (Zambon *et al*, 1985; Savitt *et al*, 1988; Maiden *et al*, 1991; Wolff *et al*, 1992; Loesche *et al*, 1992 a,b). A common finding is that the new methods were able to detect target species in subgingival plaque where traditional cultural technique failed. This has prompted calls for a review of cultural techniques as the reference or gold standard for the identification of organisms in subgingival plaque samples (Loesche *et al*, 1992 b; Loesche, 1992). These authors argue that cultural methods have multiple sources of error inherent in the procedure that compromise the reliability of this technique, and that DNA probes or immunological methods may prove to be more reliable reference standards (Loesche, 1992).

1.6 PATHOGENESIS OF PERIODONTAL DISEASE

1.6.1 Role of Dental Plaque in Periodontal Disease

It is well established that bacterial plaque forming on the teeth is the primary aetiological factor of periodontal disease (Löe *et al*, 1965; Suomi *et al*, 1971). Whereas there is excellent evidence linking dental plaque to gingivitis, the relationship between plaque and periodontitis appears to be more complex (Williams *et al*, 1992). This evidence has been derived from a variety of cross-sectional, longitudinal, human and animal studies.

Longitudinal experimental gingivitis studies in humans (Löe *et al*, 1965; Theilade *et al*, 1966) and animals (Lindhe *et al*, 1973) have shown that complete withdrawal of oral hygiene procedures results in a steady increase in plaque deposits and gingival inflammation. Reinstitution of plaque control measures results in removal of plaque and a return to gingival health within one week. Inhibition of plaque and gingivitis was achieved in a group of volunteers, after the cessation of all oral hygiene procedures by rinsing twice daily with a solution of 0.2% chlorhexidene gluconate. On discontinuation of the mouthwash, plaque formed at normal rates with the development of gingivitis again (Löe and Schiøtt, 1970). These results confirmed that dental plaque was responsible for the production of experimental gingivitis in these volunteers.

Animal studies have also been important in establishing the role of dental plaque in the aetiology of destructive periodontal disease. Longitudinal studies of ligature-induced periodontal disease in beagle dogs revealed that gingivitis developed as a result of plaque accumulation (Egelberg, 1965). If this gingivitis was maintained for five to seven months, permanent destruction of the periodontal tissues ensued that was typical of human periodontal disease (Saxe *et al*, 1967; Lindhe *et al*, 1973).

Further evidence for the role of dental plaque in chronic inflammatory periodontal disease comes from observations that improved oral hygiene procedures have been shown to reduce gingivitis (Lövdal *et al*, 1961) and, in some cases, markedly retard the progression of periodontitis (Suomi *et al*, 1971; Lindhe *et al*, 1984; Lindhe and Nyman, 1984).

1.6.2 Secondary Aetiological Factors

Secondary aetiological factors are recognised to contribute to the pathogenesis of periodontal disease and can act either locally, and influence plaque accumulation or systemically, and result in a modified host response to dental plaque.

1.6.2.1 Local factors associated with periodontal disease

Local factors that can influence periodontal disease can be divided into factors that promote plaque accumulation and those that cause reduced salivary flow. Mechanical plaque traps that promote plaque accumulation and therefore may predispose to periodontal disease include calculus, carious cavities, margins of restorations and crowns, partial dentures, orthodontic appliances, crowding and malocclusions and anatomical variations of tooth morphology. Reduced salivary flow can be produced by mouthbreathing or xerostomia, both of which may result in decreased antibacterial actions of saliva. The resulting gingivitis has been attributed to accelerated plaque accumulation (Williams *et al*, 1992).

1.6.2.2 Systemic factors associated with periodontal disease

A number of systemic factors have been proposed that are thought to modify the host response to dental plaque in a variety of ways and result in different forms of

periodontal disease. These can range from gingival hyperplasia seen in conditions associated with hormonal changes i.e. pregnancy, and drug-induced hyperplasia i.e. phenytoin, to increased or accelerated periodontal destruction which can result from defects in tissue repair i.e. Ehlers-Danlos syndrome, or defects in host defences (Williams *et al*, 1992).

1.6.2.2.1 Gingival hyperplasia

Increased progesterone levels, attributed to pregnancy, the contraceptive pill and puberty, are thought to cause vascular changes in the gingival tissues that can result in a hyperplastic, haemorrhagic gingivitis (Löe and Silness, 1963; Hugoson, 1970). The effects of some drugs or their metabolites on gingival fibroblasts is believed to cause gingival hyperplasia, however this may be reduced by good oral hygiene. The drugs believed to contribute to hyperplasia include phenytoin, cyclosporin A and nifedipine (Williams *et al*, 1992).

1.6.2.2.2 Defects in tissue repair associated with accelerated periodontal disease

Several nutritional and genetic disorders can result in defective collagen turnover, which is believed to contribute to accelerated periodontal disease. These include ascorbic acid deficiency (scurvy), protein deficiency (Kwashiorkor), hypophosphatasia and Ehlers-Danlos syndrome (Williams *et al*, 1992; Wilton *et al*, 1988).

1.6.2.2.3 Defects in host defences associated with accelerated periodontal disease

Increased periodontal destruction can to be a feature of a number of systemic conditions that often result from functional abnormalities of PMNL, although other defects are also suspected in some diseases. Conditions that can be associated with

increased periodontal disease include diabetes mellitus, acquired and genetic defects in PMNL function, and HIV infection.

1.6.2.2.3.1 Diabetes mellitus

Changes in periodontal vasculature and impairment of PMNL function have been reported in relation to increased periodontal destruction seen in poorly controlled insulin-dependent diabetes mellitus patients (Safkan-Seppälä and Ainamo, 1992). Non-insulin dependent diabetes mellitus patients are also thought to be at risk for periodontal disease (Emrich *et al*, 1991).

1.6.2.2.3.2 Defects in PMNL function

Agranulocytosis, cyclic neutropenia and chronic idiopathic neutropenia have all been associated with severe destructive periodontal disease (Zubery *et al*, 1991; Prichard *et al*, 1984; Newman and Rule, 1983; Vaughan *et al*, 1990). The severe periodontal destruction sometimes seen in patients with Down's syndrome, Papillon-Lefèrve syndrome and Chediak-Higashi syndrome (Eklund *et al*, 1968; Clark and Kimball, 1971) is believed to be related to various defects in PMNL function associated with these conditions (Wilton *et al*, 1988). A percentage of subjects infected with HIV can suffer from a range of clinically distinct periodontal diseases. These range from a linear gingivitis and ANUG to more destructive lesions i.e. HIV-periodontitis and necrotising stomatitis (Winkler *et al*, 1988). These conditions will be discussed in section 1.8.4. and the contribution of a suppressed or defective host response to periodontal disease will be discussed in section 1.6.5

1.6.3 Microbiology of Periodontal Disease

The study of the microbiology of periodontal diseases has been made difficult by an array of problems, however, considerable progress has been made in the last twenty years (Socransky *et al*, 1982, 1987).

1.6.3.1 Gingivitis

Although specific pathogens have been proposed for gingivitis (Loesche and Syed, 1978), most investigators agree that gingivitis is a non-specific inflammatory reaction to indigenous bacteria. The composition of the oral microbiota may vary from person to person and from site to site (Theilade, 1986).

1.6.3.2 Acute necrotising ulcerative gingivitis

Loesche *et al* (1982) isolated a constant flora comprising *Treponema* spp., *Bacteroides intermedius*, *Fusobacterium* and *Selenomonas* spp. in addition to a highly complex variable flora comprising of 70 taxonomic groups in plaque samples from sites of ANUG. Although it is believed that these four bacterial groups contribute significantly to the pathogenesis of ANUG, other factors including stress, fatigue, smoking, poor oral hygiene are believed to be important predisposing factors (Johnson and Engel, 1986).

1.6.3.3 Periodontitis

1.6.3.3.1 Specific and non-specific plaque hypotheses

Traditionally, there exist two main theories of the microbial aetiology of destructive periodontal disease, namely the non-specific and the specific plaque hypotheses (Socransky *et al,* 1982). Historically, these two theories were diametrically opposed, one or other dominating the opinion of oral microbiologists over the last 100 years. Proponents of the specific theory believed that there existed a single pathogenic species in destructive periodontal diseases and that treatment should be directed towards the elimination of this species. Prevention of periodontal disease could be achieved by the prevention of colonisation by the species (Theilade, 1986). Supporters of the non-specific theory of plaque believed that destructive periodontal diseases were caused by indigenous microorganisms and that all plaque bacteria contributed to the pathogenesis of the disease. Treatment of the disease should involve the elimination of plaque and prevention would be achieved via adequate plaque control (Theilade, 1986).

Improvements in microbiology, sampling techniques and characterisation of subgingival plaque samples from individual sites, as opposed to the analysis of pooled plaque samples, have revealed great complexity and variability in microbiology of plaque between individuals, between sites within the same individual and also from time to time within the same site (Moore *et al*, 1984). A number of investigators proposed that different microbial complexes were associated with different periodontal diseases (Newman *et al*, 1976; Newman and Socransky, 1977; Slots, 1976; Tanner *et al,* 1979). This idea was enhanced by the mounting evidence that *A. actinomycetemcomitans* had a major aetiological role in both juvenile periodontitis and adult periodontitis (Newman *et al*, 1976; Slots, 1976; Slots *et al*, 1980; Mandell and Socransky, 1981; Zambon *et al*, 1983b). Black-pigmenting *Bacteroides* and *spirochaetes* were also found to be

associated with destructive periodontal disease in adults (Tanner *et al*, 1979, 1984; Spiegel *et al*, 1979). Dzink *et al* (1985,1988) compared the predominant cultivable microbiota from active and inactive sites and concluded that the likelihood of a site showing activity increased dramatically if any, or a combination of any, of the following species were present in increased numbers; *A. actinomycetemcomitans, Bacteroides forsythus, Bacteroides gingivalis, Bacteroides intermedius, Wolinella recta, Peptostreptococcus micros*. These species were termed suspected periodontal pathogens. These authors also found that the presence of *Capnocytophaga ochracea, Veilonella parvula, Streptococcus mitis, Streptococcus sanguis II,* or *Actinomyces* spp. appeared to lessen the chances of a site being active and so were termed beneficial species. However, some authors pointed out that suspected periodontal pathogens were not exclusive to active periodontal disease sites, but were also found in inactive pockets, albeit in lower proportions (Theilade, 1986; Newman, 1990). Indeed, Dzink *et al* (1988) were unable to demonstrate significant differences between the microbiota of active and control sites when their data were corrected for multiple comparisons. Furthermore, Moore *et al* (1991) failed to detect any significant differences between the microbiota of active and control sites in adult periodontitis subjects, although the microbiota associated with periodontitis patients differed from that in healthy subjects.

Theilade (1986) proposed modifications to the non-specific hypothesis and highlighted the similarities between it and the modified specific plaque hypothesis (Socransky, 1977). There is general agreement that a limited number of the 300 or more potential colonisers of the periodontal pocket play an important role in destructive periodontal diseases and that the various ecological pressures within a subgingival pocket are central to the aetiology of the disease. (Dzink *et al*, 1985,1988; Theilade 1986; Socransky *et al*, 1987; Socransky and Haffajee, 1990; Newman, 1990). Some workers believe imbalances or shifts in plaque ecology, perhaps from one that was host-compatible to one containing an overgrowth of indigenous organisms having pathogenic

potential, are responsible for active episodes of destructive disease (Theilade, 1986; Newman 1990). This view is similar to the theory proposed by Socransky *et al* (1988a,b, 1991). Using PCM and cluster analysis, these authors found evidence of various microbial complexes that could be associated with healthy or diseased sites. They proposed that interactions between host and resident subgingival microbiota, and between different bacterial species could result in a series of selection pressures which, over time, influenced the composition of the microbiota in the gingival crevice. This could result in a spectrum of microbial complexes at different sites within the same subject, ranging from host-compatible to pathogenic. Pathogenic microbial complexes were generally combinations of suspected periodontal pathogens (Haffajee *et al*, 1988b; Socransky *et al*, 1988a,b, 1991). Interestingly, colonisation of a site by a suspected periodontal pathogen, although necessary, was not considered sufficient for active disease, and it was proposed that a prolonged period of latency following initial colonisation may result before it initiated active disease (Socransky *et al*, 1991). Furthermore, whereas low levels of suspected periodontal pathogens may be tolerated by the host, threshold levels had to be exceeded to initiate disease progression and that these thresholds may be lower in patients who are susceptible to disease (Haffajee *et al*, 1991; Socransky and Haffajee, 1992) These authors also proposed that a carrier state could exist with respect to the suspected periodontal pathogens in some patients, making the identification of the aetiologic agents of periodontal disease more difficult.

One of the differences between these two theories is that one refers to commensal organisms with pathogenic potential, whereas the other to suspected periodontal pathogens. However, the suspected periodontal pathogens appear capable of behaving like commensal organisms for most of the time (Socransky *et al*, 1991).

In summary, it would appear that the basis for the controversy surrounding the non-specific and the specific plaque hypotheses has evaporated (Theilade, 1986; Newman,

1990). The dominant theories of the microbial aetiology of destructive periodontal diseases centre on the ecology of the subgingival pocket (Theilade, 1986; Socransky *et al*, 1988a,b, 1991; Newman, 1990). For the majority of the time, the microbiology of the subgingival pocket is compatible with periodontal health and may offer protection against colonisation with possible pathogens (Socransky *et al*, 1991; Preus, 1990). Episodes of active periodontal disease may be caused by shifts in the ecological balance of subgingival plaque towards one with a pathogenic potential (Theilade, 1986; Newman, 1990). Shifts in the ecological balance of subgingival plaque at a given site could be caused by alterations of local enviroment such as increased supragingival plaque accumulation (Theilade 1986; Katsanoulas *et al*, 1992), successful colonisation and/or breach of tolerated threshold levels by specific species with pathogenic potential (Socransky *et al*, 1991; Socransky and Haffajee 1992). However, as indicated in section 1.4, systemic factors may contribute to the pathogenesis of periodontal disease and shifts in the ecological balance of the subgingival microbiota may also occur due to the effects of systemic broad-spectrum antimicrobials (Slots *et al*, 1988; Rams *et al*, 1992; Helovuo *et al*, 1993), or immunosuppression resulting from systemic disease or its treatment (Samaranayake *et al*, 1984; Dreizen *et al*, 1986; Tenovuo *et al*, 1990; Slots and Rams, 1991).

Whatever the cause of the change or shift in microbiology resulting in an active periodontal disease site, some of the destructive processes are thought to be mediated via the possession of virulence factors by periodontal bacteria (Theilade, 1986; Socransky and Haffajee, 1991, 1992).

1.6.3.3.2 Exogenous pathogens

Genco *et al* (1988), proposed that two suspected periodontal pathogens could be considered entirely exogenous pathogens, namely *P. gingivalis* and

A. actinomycetemcomitans. These workers maintain successful prevention of at least some destructive periodontal disease may be attained by prevention of the spread and transmission of *A. actinomycetemcomitans*. Although conclusive evidence has still to be produced, there has been renewed interest in the acquisition and transmission of *A. actinomycetemcomitans* and other periodontal bacteria (Fisken, 1990; Könönen *et al*, 1992 a,b; Van Steenbergen *et al*, 1993).

1.6.4 Microbial Mechanisms in the Pathogenesis of Destructive Periodontal Diseases

1.6.4.1 Virulence factors

Virulence factors may be defined as the set of unique properties of a species which permit colonisation, evasion of host defences and cause tissue damage (Slots and Genco, 1984; Theilade, 1986; Socransky and Haffajee, 1991). Theilade (1986) argued that all plaque bacteria contributed, more or less, to the destructive disease process by the possession of various virulence factors, however, certain species played a larger role than others because they increased in both number and proportion during active disease in addition to virulence factors, mainly to evade host defences. Others proposed that only pathogenic species would have multiple virulence factors (Socransky and Haffajee, 1991). In reviewing the literature, these authors listed a number of mechanisms whereby periodontal bacteria could contribute to the destructive process in periodontal disease. Among the factors believed to aid colonisation are adhesins, which are specific molecules on the surface of bacteria that allow attachment to host cells or the tooth surface. These molecules are also thought to play a role in allowing bacteria to attach to one another, a process called coaggregation. Evasion of the host defences by bacteria could be mediated by the production of proteases to IgG and IgA, changing surface antigens or mimicking host antigens. In addition to leukotoxin, produced by

A. actinomycetemcomitans and the capsules produced by *P. gingivalis*, a number of species have developed ways of disrupting phagocytosis and PMNL-mediated killing. Mechanisms of suppression of the immune system involving both T and B lymphocytes and activation of T-suppressor lymphocytes have been demonstrated. Invasion of host tissues by bacteria in destructive periodontitis has been demonstrated, although it remains a point of controversy and is not universally accepted as a common event in the process of tissue damage (section 1.7). However, tissue damage could result solely from the presence of specific bacteria or their products, or both, in the periodontal tissues or from an immunological reaction to them. Virulence factors which cause tissue damage can be divided in to those that cause damage directly (e.g. hydrogen sulphide), substances that cause host cells to release biologically active factors (e.g. lipopolysaccharide) and substances that degrade the intercellular matrix (e.g. collagenase).

Socransky and Haffajee (1991) concluded that microbial virulence factors could adversely affect virtually any cell or macromolecule present in the periodontium. However, due to the fact that periodontal disease activity was rare, they suggested that these substances are often not produced, do not react with their target or are prevented from acting in the pocket environment. This suggestion would seem to gain support from the observation that not all clonal types of a pathogenic species are virulent (Finlay and Falkow, 1989). Clonal variants of periodontal pathogens with different virulence have been isolated (Grenier and Mayrand 1987; McKee *et al*, 1988; Neiders *et al*, 1989) and have been proposed as a possible explanation to the observed variation in disease between sites and between patients (Socransky and Haffajee, 1992). Another possibility is that the production of virulence factors by pathogenic species may be turned on or off in response to physical changes in the enviroment, for example, temperature, osmolarity or metal ion concentration, including Fe^{3+}, Mg^{2+}, and Ca^{2+}. Physical changes in the local enviroment may be produced by a traumatic event, such as

food impaction and result in the production of virulence factors by these dormant pathogens (Socransky and Haffajee, 1991, 1992). Finally, as mentioned above threshold levels of suspected pathogens may be needed to initiate disease progression and these thresholds may be lower in patients who are susceptible to disease (Haffajee *et al*, 1991).

1.6.5 The Host Response in Periodontal Disease

A number of studies published in the 1970's demonstrated the presence of host immune and inflammatory cells in diseased periodontal tissues in response to bacterial plaque e.g. Page and Schroeder (1976, section 1.3). Research by several groups has indicated that an immune response, directed against specific bacteria present in the subgingival pocket, is elicited by patients with periodontal disease. These responses vary between individuals and are under genetic control (Seymour, 1991). Subgingival bacteria together with soluble products (i.e. enzymes and toxins) and extracellular matrix are all antigenic and, as with other bacterial diseases, mainly stimulate a humoral immune response (Williams *et al*, 1992). There are a number of ways in which specific antibodies to these elements can be produced. Antigens that penetrate the epithelial barrier into the gingival tissues are recognised by macrophages, processed and taken to local lymph nodes where they are presented to B-lymphocytes with the help of T-helper lymphocytes ($CD4^+$ T-cells). The B-lymphocytes that recognise each individual antigen undergo blast transformation and differentiate into plasma cells. These cells remain in the lymph node and secrete specific antibody into the blood stream which is then carried back to the gingival tissues, passes into the gingival inflammatory exudate and into the gingival crevice via GCF. Approximately 75% of the antibody produced in this way is IgG with about 7% being IgM. However, some of the antibody is produced by local plasma cells that remain in the gingival tissues and these cells account for approximately 15% of the total IgG produced (Ebersole *et al*, 1985; Tew *et al*, 1985). Although IgG

subclasses predominate local antibody production (Mackler *et al,* 1978; Ogawa *et al,* 1989) some IgA and IgM producing cells have also been demonstrated (Mackler *et al,* 1977; Genco *et al,* 1974). There is also evidence that some bacterial products act as polyclonal activators of B-cells which causes differentiation of these cells into plasma cells and results in the production of irrelevant antibody that is secreted locally (Tew *et al,* 1989). Another mechanism of specific antibody production involves Gut Associated Lymphoid Tissue which recognises bacterial antigens swallowed with saliva and results in the secretion of specific IgA with saliva. Specific antibodies can be effective against periodontal bacteria by opsonisation, activating complement, activating PMNL enzyme secretion, binding to bacteria and therefore inhibiting attachment to host surfaces and inhibiting bacterial metabolism directly. They are also believed to be able to neutralise toxins and inhibit enzymes produced by bacteria (Williams et al, 1992).

1.6.5.1 Bacterial factors

One of the most intensively studied examples of host-bacterial interaction in periodontal disease is the association between *A. actinomycetemcomitans* and LJP. This organism is detected frequently and often in high numbers in LJP lesions (Newman *et al,* 1976; Slots, 1976; Zambon *et al,* 1983a,b), correspondingly the majority of LJP patients have elevated serum antibodies to this species (Ebersole *et al,* 1980; Altman *et al,* 1982). Furthermore, local antibody production has also been observed and antibodies have been detected in gingival crevicular fluid (Murray and Genco, 1980; Ebersole *et al,* 1985; Tew *et al,* 1985).

A. actinomycetemcomitans is believed to have developed a number of strategies to evade host responses (Slots and Genco, 1984; Socransky and Haffajee, 1991). It has a capsule which resists opsonisation and phagocytosis and has its LPS with potent bone resorbing activity. It also has a number of tissue degrading enzymes which are

transported extracellularly in membrane vesicles. More importantly, *A. actinomycetemcomitans* can also secrete a leucotoxin that is believed to effect PMNL and macrophages (Baehni *et al*, 1979). However, there appears to be considerable interplay between this species and the host defences (reviewed by Genco, 1992). The host is believed to overcome these difficulties by first producing specific antibodies to neutralise the leukotoxin (McArthur *et al*, 1980; Tsai *et al*, 1981). However as this is not sufficient to eliminate *A. actinomycetemcomitans* the host then produces opsonic antibodies which fix complement and prepare the organism for phagocytosis and intracellular killing by phagocytes (Baker and Wilson, 1989). Zambon *et al* (1983b) identified three distinct serotypes of *A. actinomycetemcomitans*, however results of recent work by Gmür *et al* (1993) have indicated that there may be as many as five different serotypes. These serotypes are believed to have different virulence potential with only certain strains possessing leucocidal activity and it is these strains that are thought to predominate in LJP subjects (Baehni *et al*, 1980; Asikainen *et al*, 1991b).

Many patients with advanced periodontal disease have been shown to elicit serum IgG responses to *P. gingivalis* and *P. intermedia* (Mouton *et al*, 1981; Ebersole *et al*, 1986). *P. gingivalis* is also thought to possess a number of virulence factors (Slots and Genco, 1984; Okuda and Takazoe, 1988; Socransky and Haffajee, 1991). It has a capsule that resists opsonisation by complement, phagocytosis and killing by PMNL in addition to LPS which inhibits chemotaxis and killing by leucocytes. However, *P. gingivalis* has a wide range of tissue degrading enzymes including proteases, fibrinolysins and phospholipases. Interestingly, it sheds small membranes vesicles, containing protease, that are believed to be small enough to pass into host tissues (Slots and Genco, 1984; Okuda and Takazoe, 1988). *P. intermedia*, however has a less impressive array of virulence factors than *P. gingivalis* but it does have a capsule that resists phagocytosis and LPS which is believed to affect immune cells (Williams *et al*,

1992). Finally, prepubertal periodontitis patients have been found to have elevated systemic antibody responses to *C. sputigena*, whereas post-pubertal periodontitis patients had a reduced response to this organism (Vandesteen *et al,* 1984).

Longitudinal measurements of serum antibodies in periodontitis patients have indicated changes in levels following treatment (Haffajee *et al,* 1984). Furthermore, Ebersole *et al* (1985, 1986, 1987) demonstrated that the distribution of elevated GCF antibodies and the presence of corresponding organisms at the same sites agreed in up to 78% of samples. These authors also found a strong correlation between the isolation of specific organisms from subgingival plaque at active sites with high levels of serum antibodies to the same organisms (Ebersole *et al,* 1987). Recent work by Powell *et al* (1991) demonstrated levels of IgG_4 in GCF are elevated in GCF compared to levels in serum and suggested that IgG_4 production was induced locally. However, Gunsolley *et al* (1987), found that levels of serum antibody to *P. gingivalis* and *A. actinomycetemcomitans* were inversely related to disease level in a sample of periodontitis patients, and postulated that a failure to mount a substantial antibody response to these organisms could also result in more widespread disease in some patients. Similarly, Kinane *et al* (1993) found GCF levels of specific IgG to *P. gingivalis* were significantly lower in chronic periodontitis patients with deeper pockets and more gingival inflammation.

Shenker (1987) questioned the effectiveness of the host's immune response, whether humoral or cell-mediated, in periodontal diseases as infection with specific bacteria seems to persist despite an apparently normal immune response. He and his colleagues demonstrated that immunosuppressive characteristics, both B and T-lymphocyte activation inhibition, were possessed by a number of suspected periodontopathogens including *T. denticola, A. actinomycetemcomitans*, *F. nucleatum* and several

Bacteroides ssp (Shenker *et al*, 1982 a,b, 1984 a,b). The leucotoxic properties of strains of *A. actinomycetemcomitans* have been mentioned above.

1.6.5.2 Polymorphonuclear leucocytes

Normal function and numbers of circulating PMNL are regarded as critical to the maintenance of periodontal health (Miller *et al*, 1984; Page, 1990) as they possess a considerable number of powerful antimicrobial mechanisms (Miyasaki, 1991). The importance of PMNL in the host response may be gauged by observations that patients with neutrophil disorders i.e. agranulocytosis, cyclic neutropenia and chronic idiopathic neutropenia suffer from severe periodontal disease (Aubrey and Hibbard, 1973; Zubery *et al*, 1991; Baehni *et al*, 1983; Prichard *et al*, 1984; Vaughan *et al*, 1990). Furthermore, patients with systemic diseases that can result in reduced neutrophil function i.e. poorly controlled diabetes and Down's syndrome, have also been found to be at risk for severe periodontal disease (Emrich *et al*, 1991; Safkan-Seppälä and Ainamo, 1992). Interestingly, several rare syndromes in which abnormal PMNL functions are a feature can also suffer from severe periodontal disease i.e. Chediak-Higashi Syndrome, Papillon-Leférve Syndrome, Lazy Leucocyte Syndrome. For example, Chediak-Higashi Syndrome, which is transmitted by an autosomal recessive gene, is associated with PMNL functional defects including reduced chemotactic response and defective intracellular killing of bacteria. This syndrome is characterised by an increased susceptibility to bacterial infections including advanced periodontal disease (Eklund *et al*, 1968; Clark and Kimball, 1971).

There is also some evidence that patients with severe periodontal disease, but who are otherwise healthy, can have altered PMNL function. PMNL function in LJP patients has been studied extensively, however the exact nature of the defects in these patients remain an area of considerable debate. Both defective phagocytosis and chemotaxis

have been demonstrated in PMNL from some LJP patients (Cianciola *et al*, 1977; Suzuki *et al*, 1984; Clark *et al*, 1977). Van Dyke *et al* (1981, 1985a,b, 1987) reported that the chemotactic defect is associated with reduced numbers of chemoattractant receptors on the PMNL cell surface and produced evidence that this defect may be familial. Interestingly, altered PMNL function is also thought to contribute to ANUG, rapidly progressive periodontitis and refractory periodontitits (Cogan *et al*, 1983: Genco *et al*, 1986; Leone *et al*, 1987; Van Dyke *et al*, 1985a; Van Dyke and Hoop, 1991). Nonetheless, PMNL and their contents are considered major contributors to the acute inflammatory process observed in active adult periodontitis and may be responsible for some host tissue damage seen in active periodontal disease (Kryshtalskyj *et al*, 1986; Lamster *et al*, 1991).

1.6.5.3 Lymphocytes

Early phenotypic studies, using enzyme and cell surface antigen markers by Mackler *et al* (1977) and Seymour and Greenspan (1979), reported that the majority of lymphocytes present in active periodontitis lesions were B-lymphocytes, whereas stable, gingivally confined lesions were dominated by a T-lymphocyte infiltrate, as seen in gingivitis occurring in children (Seymour *et al*, 1981). The hypothesis that active tissue destruction was associated with the conversion of a stable T-lymphocyte lesion to a B-lymphocyte/plasma cell lesion was challenged by Page and Schroeder (1982) who suggested that B-lymphocyte dominated lesions could also remain stable for prolonged periods. However, recently Stoufi *et al* (1987) and Johannessen *et al* (1990) found that whereas both T-cells and B-cells could predominate lesions, there was considerable variation in the numbers of plasma cells present in individual active lesions.

Ivanyi and Lehner (1970, 1971) reported that peripheral blood lymphocytes (PBL) from adult periodontitis patients underwent enhanced blastogenesis, compared to PBL from

healthy controls, when stimulated by extracts of *Veillonella alcalescens* and *Actinomyces viscosus*. However, later studies, found that PBL from periodontitis patients behaved normally, in terms of blastogenesis, when presented with bacterial antigens, mitogens or polyclonal activators (Kiger *et al*, 1974; Donaldson *et al*, 1982). Interestingly, in a review of recent work, Tew *et al* (1989) proposed that polyclonal B-cell activation is increased in some patients with periodontitis but estimated that approximately 30% of subjects with severe periodontal disease do not have this response. So at present the significance of polyclonal B-cell activation in the pathogenesis of periodontitis, and whether it resolves after treatment, are still unclear.

Both T-helper ($CD4^+$) and T-suppressor ($CD8^+$) cell subsets are present in periodontitis lesions (Çelenligil, 1990) and their activity in periodontitis is inferred by demonstrations of increased expression of HLA-DR antigens and increased concentrations of IL-1 in both GCF and gingival tissue extracts from periodontitis patients (Reinhardt *et al*, 1988; Masada *et al*, 1990; Hönig *et al*, 1989). However a depressed $CD4^+$: $CD8^+$ ratio has been found in periodontal lesions compared to that seen in health or in peripheral blood (Okada *et al*, 1982; Taubman *et al*, 1984; Manti *et al*, 1984). This ratio is believed to be result of a local reduction in the numbers of $CD4^+$ T-cells (Taubman *et al*, 1984; Stoufi *et al*, 1987) and it is thought that many of the $CD4^+$ T-cells that are present in periodontal lesions are in fact memory T-cells (Taubman *et al*, 1991). These authors also proposed that local immunoglobulin and antibody production could be suppressed by gingival $CD8^+$ T-cells and aided by gingival macrophages. Interestingly, Kinane *et al* (1989) observed significantly depressed $CD4^+$: $CD8^+$ T-cell ratios in peripheral blood of 12 patients with early onset periodontitis compared to age and sex matched controls. Collectively these studies indicate that depressed $CD4^+$: $CD8^+$ T-cell ratio at a local level may contribute to the pathogenesis of periodontal disease. The association between HIV infection,

characterised by falling $CD4^+$ and rising $CD8^+$ T-cell numbers, resulting in depressed $CD4^+$: $CD8^+$ T-cell ratios, and periodontal disease will be discussed in section 1.8.4.

Peripheral blood lymphocytes will undergo spontaneous proliferation after 5 to 7 days in the absence of exogenous stimulants. This is called the autologous mixed lymphocyte reaction (AMLR) and can be indicative of immunoregulatory activity by T-lymphocytes. The AMLR has been shown to be depressed in patients with advanced periodontal destruction in a number of studies (Tew *et al*, 1983; Suzuki *et al*, 1983, 1984). However this returned to normal after treatment, suggesting that the depressed response may be the result of disease activity, rather than the cause.

1.6.5.4 Monocytes and macrophages

It has been known for some time that macrophages play an important role in phagocytosis and antigen presentation to lymphocytes, however, in addition it was recently proposed that they may also help regulate local antibody production (Taubman *et al*, 1991). In a recent study, McFarlane *et al* (1990) demonstrated that cultured peripheral blood monocytes from periodontitis patients released greater quantities of IL-1β and TNF-α than monocytes from healthy subjects when stimulated by bacterial lipopolysaccharide (LPS). Whereas, Garrison and Nicols (1989) demonstrated increased PGE_2 release from monocytes of adult periodontitis patients stimulated by LPS. These studies suggest that hyper-reactivity of monocytes to LPS may place patients at risk for severe periodontal disease.

1.6.5.5 Inflammatory and immunologic mediators

The role of inflammatory and immunologic mediators in periodontal disease were reviewed by Genco (1992). The identification of numerous cytokines, produced

particularly by lymphocytes and macrophages, and the demonstration of many of these cytokines in the GCF or gingival tissue, have led to intense speculation about their contribution to the periodontal disease process (reviewed by Page, 1991). Although there are a range of cytokines, IL-1 has attracted considerable attention after it was demonstrated in GCF during periods of inflammation (Charon *et al*, 1982). Dewhirst *et al* (1985) demonstrated that osteoclast activating factor was infact IL-1β and it has since been localised in, and extracted from gingival tissue (Hönig *et al*, 1989; Jandinski *et al*, 1991). IL-1 is produced by activated macrophages and lymphocytes, platelets, endothelial cells and fibroblasts and is also thought to be autostimulatory. However, IL-1 is pleotrophic and in addition to stimulating osteoclastic-dependent bone resorption, it is also believed to mediate many processes including antigen presentation, PGE_2 release from macrophages, attachment of PMNL and monocytes to epithelial cells and induction of metalloproteinases e.g. procollagenase (Genco, 1992). IL-2, previously known as T cell growth factor is produced by activated $CD4^+$ T cells and mediates T cell growth and proliferation, and TNF-α production by activated macrophages. IL-6 is believed to regulate plasma cell infiltrate and preferentially stimulates IgG-producing plasma cells, in addition to inducing IgG_3 and IgG_4 producing plasma cells (Genco, 1992). In addition to these individual properties many cytokines including IL-1, TNF-α, IL-6 and TGF-β are thought to be important in bone remodelling.

Page (1991) summarised the role of cytokines in a possible sequence of events leading to periodontal tissue destruction: with the onset of vasculitis of small blood vessels adjacent to the junctional epithelium, bacterial components and host cytokines induce the expression of intercellular adhesion molecule, ICAM-1, on the surface of endothelial cells. LTB4, TNF-α and activated complement components induce the expression of adhesion molecules on PMNL and monocytes, which subsequently adhere to endothelial cells via ICAM-1, then move into the surrounding connective tissue.

Increased vascular permeability allows cells (PMNL, B and T-lymphocytes, macrophages) to enter the tissues. Thus bacterial components appear to initiate a sequence of events, but host factors are responsible for prolonging and amplifying the process.

PGE_2 has been the focus for research into the role of inflammatory mediators in destructive periodontal disease for some time following the demonstration of this arachnodonic acid metabolite to induce bone resorption in vitro (Genco, 1992). Furthermore, Offenbacher *et al* (1986) demonstrated that PGE_2 levels in GCF correlate well periods of active periodontal disease.

Soft tissue degradation seen in periodontal disease are thought to be caused by both bacterial and host derived enzymes i.e. plasmin, elastase and matrix metalloproteinases. Matrix metalloproteinases include collagenase, stromalysins, gelatinase and their production can be stimulated by bacteria directly or indirectly via the stimulation of host cells to produce cytokines (Genco, 1992).

1.6.5.6 Complement system

The complement cascade when activated has a number of actions that are central to control of the inflammatory response. There are two pathways in which the system may be activated and result in the release of active components. The classical pathway involves activation by antibody-antigen complexes i.e. antibody bound to the surface of bacteria, or by the alternative pathway in which complement is activated by endotoxin or by directly binding to a bacterial cell wall. Active components of complement are important in opsonisation to enhance phagocytosis by PMNL and macrophages, causing mast cells to degranulate thereby releasing histamine and other vasoactive molecules into the area to affect the vascular response in inflammation and finally by causing lysis

of some species directly. Several studies have shown that in most periodontal diseases complement activation occurred via the alternative pathway, however in LJP complement activation could have occurred via the classical and, possibly, the alternative pathway (Genco, 1992).

1.6.5.7 Hypersensitivity reactions in periodontitis

Type I, II, and III immediate hypersensitivity reactions are antibody-mediated whereas type IV hypersensitivity is cell-mediated. IgE-bearing mast cells are detected in very low numbers in periodontal tissues and therefore Type I reactions are thought unlikely to contribute to periodontal disease (Zachrisson *et al*, 1968; Nisengard, 1974). Type II reactions, in which cell lysis is mediated by complement-fixing IgG or IgM antibodies, have not been convincingly demonstrated in periodontal tissues (Ranney, 1991), whereas, fixed immune complexes, characteristic of Type III reactions, have not been observed (Genco *et al*, 1974). Type IV, cell-mediated delayed hypersensitivity reactions are also thought to be unlikely because of the predominance of B-lymphocytes in many periodontitis lesions (Ranney, 1991).

1.6.5.8 Autoimmunity in periodontal disease

Conclusive evidence of autoimmune tissue destruction in periodontal disease is lacking, despite the existence of indirect evidence of this phenomenon. Hirsch *et al* (1989) detected rheumatoid factor-producing cells in gingival tissue taken from adult periodontitis lesions. Tew *et al* (1989) suggested that polyclonal B cell activators, such as bacterial mitogens, might stimulate the proliferation of autoreactive B cell clones. Local IgG antibodies to type I collagen were demonstrated in adult periodontitis patients' tissues by Anusaksathien *et al* (1990). Singh *et al* (1990) could not demonstrate a significant difference between autoantibody levels to 11 self-antigens in

periodontitis patients and control subjects. Anusaksathien and Dolby (1991) reviewed the literature on this subject and concluded that the autoantibodies found in diseased periodontal tissue are derivatives of pre-existing natural antibodies (IgM class switched to IgG) formed during the disease process in order to remove dead cells. However, in certain circumstances this response may become excessive and lead to further tissue damage.

1.6.6 Summary of Immune Responses in Different Forms of Periodontitis.

1.6.6.1 Adult periodontitis

In his extensive review, Ranney (1991) concluded that patients with adult periodontitis had normal immune functions which were fundamentally protective against local invasion and septicaemia, since immunocompromised animals and humans often experience severe periodontal tissue destruction. Some damage to the host tissues is inevitable in the face of the inflammatory and immune mediators attracted to and released into the periodontium. Ogawa *et al* (1989) postulate that chronic exposure of B lymphocytes in gingival tissues to antigen during periodontitis induces a switch from production of IgG_1 (which activates complement) to IgG_4 (which does not), hence host-mediated damage may be gradually reduced over time.

1.6.6.2 Early onset periodontitis

1.6.6.2.1 Localised juvenile periodontitis

A number of studies have produced conflicting evidence about the immune status of patients with LJP (Lehner *et al*, 1974; Suzuki *et al*, 1984). The prevailing opinion now

however, is that humoral and cell-mediated responses to *A. actinomycetemcomitans* are normal, but PMNL function is abnormal in many, but not all, of these patients (Cianciola *et al*, 1977; Clark *et al*, 1977; Van Dyke *et al*, 1981, 1984, 1985b; Page *et al*, 1985). Infection by highly virulent organisms will also contribute to the disease process. A good antibody response to *A. actinomycetemcomitans* seems to reduce the extent and severity of EOP (Ranney *et al*, 1982; Gunsolley *et al*, 1987).

1.6.6.2.2 Rapidly progressive periodontitis and generalised juvenile periodontitis

These disease categories do seem to be associated with immunological abnormalities in the host. Engel *et al* (1984) demonstrated B lymphocyte hyper-responsiveness to mitogens in cells from patients with severe, generalised EOP. Suzuki *et al* (1984) found that a depressed AMLR was associated with generalised EOP and RPP, but not with LJP. As mentioned above, this may be the result of disease, not a precipitating factor. Low serum antibody titres to *A. actinomycetemcomitans* and *P. gingivalis* have been associated with generalised EOP, as opposed to high titres to these species in patients with localised disease (Gunsolley *et al*, 1987). This may be due to the immunosuppressive abilities of certain isolates of these bacteria (Shenker, 1987). The lack of an effective immune response seems to permit a much more rapid and destructive process to occur.

1.6.6.2.3 Prepubertal periodontitis

Page *et al* (1983) reported chemotactic defects in both PMNL and monocytes in two cases of generalised prepubertal periodontitis patients, whereas defects in chemotaxis were reported to involve only PMNL in three cases of localised prepubertal periodontitis.

1.7 CURRENT CONCEPTS AND DIFFICULTIES IN THE STUDY OF PERIODONTAL DISEASES

1.7.1 Indicators of Periodontitis

Although periodontal disease can be associated with a range of clinical signs, e.g. gingival inflammation, bleeding, accumulation of plaque, these represent variable responses between individuals. Of all the clinical signs associated with the disease only two can be considered as a reliable indicators of periodontitis, namely localised attachment loss and localised alveolar bone loss (Goodson, 1990). Methods of measurement of attachment loss rely on the detection of the base of the periodontal pocket and a fixed reference point i.e. the amelocemental junction. The degree of periodontal attachment loss at a given site is assumed to be the distance between these two points. However, a number of factors can influence this measurement including probing force, shape and angulation of probe, degree of inflammation present at the site together with the depth of the site to be measured (Blomqvist, 1986; Anderson *et al*, 1991; Mombelli *et al*, 1992; Atassi *et al*, 1992). The use of constant force manual probes in combination with stents and the advent of automated probes have attempted to address some of these problems (Van der Velden and de Vries, 1978; Jeffcoat *et al*, 1986; Gibbs *et al*, 1988; Espeland *et al*, 1991), however the accuracy and reproducibility of measurements varies greatly depending on the method used (Goodson, 1990; Mintzer and Derdivanis, 1993). As a result of these developments, error in measurement of attachment loss by traditional methods using a conventional periodontal probe has been criticised as being unacceptably high (Best *et al*, 1990; Espeland *et al*, 1991).

Methods of measurement of localised bone loss involve the use of radiographs and there are a number of techniques that can measure height of alveolar bone on a

radiograph including direct visual measurement, subtraction radiography and computer-enhanced image analysis (Lamster and Karabin, 1992). However, variations in the positioning and angulation of film, exposure and film processing can also produce errors in these measurements (Webber *et al*, 1990; Hausmann *et al*, 1991).

A common problem with both direct measurement of attachment level and radiographic assessment of alveolar bone loss is that a single measurement is indicative only of the sum total of past periodontal disease experience at any given site. Two examinations are required to determine whether further periodontal attachment loss has occurred between the examinations. This implies that periodontal disease progression can only be determined retrospectively using these methods of measurement (Goodson, 1990, 1992).

1.7.2 Models of Periodontal Disease Progresion

Cross sectional epidemiological data from a variety of geographic locations (reviewed by Goodson, 1990) indicated that the correlation between age and severity of periodontal disease was linear. Although there were wide individual variations, the mean rate of periodontal destruction was estimated between 0.1 and 0.2 mm per site per year. The results of these studies gave rise to the continuous model of disease progression in which periodontal attachment loss progressed in diseased sites in a slow, inexorable fashion in the absence of treatment. However, a number of longitudinal studies of periodontal attachment loss in individual sites in groups of patients with prior evidence of periodontal disease challenged the continuous model of disease progression (Goodson *et al*, 1982; Haffajee *et al*, 1982; Lindhe *et al*, 1983; Socransky *et al*, 1984). These studies found that attachment loss occurred in only a small percentage of sites monitored, but when observed the magnitude of loss was usually between 2 - 5 mm. The majority of sites, despite the presence of inflammation showed no progression.

These observations in conjunction with results from animal studies, prompted the theory that disease progression was more likely to occur in rapid bursts of activity at only a few sites, interspersed with long periods of quiescence (Socransky *et al*, 1984). Furthermore, it was proposed that these bursts of activity could occur randomly throughout adult life i.e. Random Burst Theory, or that they would be more likely at certain periods of an individuals life i.e. Asynchronous Burst Theory. According to these theories active disease is believed to be an uncommon event occurring in 5% of sites of patients with untreated periodontal disease (Goodson *et al*, 1982; Haffajee *et al*, 1983). Long term periodontal health is associated with a stable host compatible subgingival microbiota with infrequent active phases of the disease brought quickly under control by host and/or microbial influences (Socransky *et al*, 1988a).

Recently, however, the use of automated probes, with the capacity to measure attachment levels to an accuracy of 0.2 mm, have found that the prevalence of disease progression is dependent on the threshold of attachment loss used to indicate active disease (Jeffcoat and Reddy, 1991). These authors found that active disease occurred in 29% of sites measured when a threshold of 0.4 mm was used, compared to 2% of sites deemed active when the threshold was over 2.4 mm. Similar results using subtraction radiography (Deas *et al*, 1991) have resulted in renewed debate on the rate of active disease and patterns of disease progression (Jeffcoat and Reddy, 1991; Goodson, 1992).

1.7.3 Tests for Periodontal Disease Activity

As indicated above, a single measurement of attachment level or bone loss cannot give an indication if a site is undergoing active periodontal destruction at any one point in time (Greenstein and Caton, 1990). Therefore there has been considerable interest in recent years in developing a test, preferably chairside, that could indicate active

periodontal disease. These were reviewed by Reddy and Jeffcoat (1993) and fall into two categories. The first involves methods to identify putative periodontal pathogens (reviewed in section 1.5), whereas the second involves the search for markers of inflammation or tissue breakdown in gingival crevicular fluid. These include levels of host-derived enzymes i.e aspartate aminotransferase, collagenase, β-glucuronidase, lactate dehydrogenase, arylsulphatase and elastase; tissue breakdown products i.e. hydroxyproline and glycosaminoglycans, and inflammatory mediators i.e. TNF-α , IL-1β, PGE_2 (Offenbacher *et al*, 1986; Rossomando *et al*, 1990; Stashenko *et al*, 1991; Johnson, 1991). Recently, temperature of the gingival sulcus has been investigated as a possible indicator of active periodontal disease (Haffajee et al, 1992b,c).

1.7.4 Risk Indicators for Periodontal Disease

There has also been interest in the possibility of identifying individual sites or subjects that are at risk for destructive periodontal disease (Bader *et al*, 1990; Beck *et al*, 1990; Grbic *et al*, 1991; Haffajee *et al*, 1991a,b). Some of these studies have shown that age and history of previous disease are two important risk factors for progressive disease. Other factors are thought to include tobacco use, immunosuppression, stress, oral hygiene, genetics, poorly controlled diabetes and specific microbiology (Bader *et al*, 1990; Reddy and Jeffcoat, 1993).

1.7.5 Microbiological

Difficulties in the search for pathogens of destructive periodontal disease were reviewed by Socransky *et al* (1987).

1.7.5.1 Sampling strategy

Problems in detecting periods of disease activity will have a significant effect on the timing of sampling a periodontal pocket for microbiological investigation (Socransky *et al*, 1987). If active disease is an uncommon event and imbalances in subgingival microbiota are quickly brought under control, then the chances of sampling a periodontal site at the peak of disease activity will be low (Theilade, 1986; Socransky *et al*, 1987; Sterne *et al*, 1990). Therefore, microbiological results of samples from sites of uncertain disease status may lead to either an under estimation of the contribution of a suspected periodontal pathogen to the disease process, or simply reflect the microbiota of a recovering disease site (Socransky *et al*, 1987).

The choice of sampling technique and handling of the samples can have a profound effect on the recovery of microorganisms from a subgingival pocket. The most common methods of obtaining a subgingival plaque sample from a site involve either the use of a sterile scaler or paper point. Some workers have reported better results with a scaler compared to paper point (Tanner and Goodson, 1986; Baker *et al*, 1991), whereas others have favoured the paper point (Dahlén *et al*, 1990; Renvert *et al*, 1992). However, it is clear that caution should be exercised when comparing results from papers that use dissimilar sampling methods (Tanner and Goodson, 1986, Baker *et al*, 1991; Wikström *et al*, 1991).

These difficulties are superimposed on the concerns of some workers that dispersal methods and selection pressures of non-selective media used in cultivable techniques, distort the microbiological profile of a plaque sample leading to undue attention being focused on the cultivable organisms only (Socransky *et al*, 1987; Theilade, 1986). These factors, in addition to the increased sensitivity claimed by new rapid methods of bacterial identification, have led to calls for the replacement of culture techniques as the gold standard of bacterial identification in subgingival plaque samples (Loesche *et al*, 1992a,b,c).

1.7.5.2 Virulence

Asikainen *et al* (1991b) used indirect immunofluorescence microscopy to assess the prevalence of different serotypes of *A. actinomycetemcomitans* between healthy, LJP, EOP and adult periodontitis sites. They found various serotypes of *A. actinomycetemcomitans* in 96 - 100% of the sample groups, however, the majority of patients had only one serotype and this varied between the groups studied. Healthy sites were more likely to colonised by serotype c and diseased sites were more likely to be colonised by serotype b. These authors proposed that different virulence could be attributed to the different serotypes of *A. actinomycetemcomitans*. This is in agreement with DiRienzo and Slots (1990) who proposed that varying virulence potential may exist with different serotypes of *A. actinomycetemcomitans*.

1.7.5.3 Invasion of host tissues

Microscopic techniques have demonstrated the presence of bacteria within periodontal tissues in ANUG (Listgarten, 1965; Courtois *et al*, 1983) in LJP (Saglie *et al*, 1982) and in adult periodontitis (Allenspach-Petrzilka and Guggenheim, 1983). These findings prompted speculation that active bacterial invasion could result in acute episodes of

periodontal destruction (Allenspach-Petrzilka and Guggenheim, 1983). However, some workers believe that, with the exception of ANUG and possibly LJP, active bacterial invasion has still to be convincingly demonstrated in periodontal disease (Listgarten, 1986; Liakoni *et al*, 1987; Newman, 1990). They argue that the majority of previous studies have shown only a haphazard distribution of a relatively small number of bacteria in the periodontal tissues in a minority of adult periodontitis patients and therefore invasion is unlikely to be a major factor in the pathogenesis of periodontitis. These authors propose that it is more likely that bacteria were passively introduced into the tissues for example as a result of minor trauma during mastication.

1.7.6 Statistical Analysis

The difficulties in statistical analysis of periodontal research data were reviewed by Sterne *et al* (1990). One of the problems encountered is the site versus subject controversy, which arises because periodontal data is hierarchical i.e. diseased sites within subjects. On the one hand, periodontal disease is observed to occur at individual sites, however site-based analyses are inappropriate because sites within an individual are not independent. Furthermore, the number of measurements made for each subject i.e. over 100 for attachment level alone, and the number of bacterial species being investigated can create problems in analyses if corrections for multiple comparisons are not made (Dzink *et al*, 1988; Sterne *et al*, 1990). Analyses could be made simpler if a suitable summary statistic for each variable was found and statistical comparisons made at a subject level. Although statistically acceptable, this approach results in a considerable loss of detail and has been criticised for not being sensitive enough (Socransky *et al*, 1987). Statistical analyses used in this thesis are described in Materials and Methods.

1.8 HIV INFECTION

1.8.1 AIDS and HIV

The Acquired Immunodeficiency Syndrome (AIDS) was first described in the USA in 1981 (CDC, 1981a). Early reports suggested the disease affected male homosexuals and included opportunistic infections such as *Pneumocystis carinii* pneumonia and a previously rare tumour, Kaposi's sarcoma (CDC, 1981a,b). It was already known that such illnesses could occur in renal transplant patients and patients undergoing immunosuppressive therapy, so an underlying immune deficiency was suspected as the cause of these conditions in homosexual males. However it was not until 1984 that a retrovirus was isolated from patients with AIDS and AIDS-related complex (ARC) and recognised as the aetiologic agent of the syndrome (Gallo *et al*, 1984; Levy *et al*, 1984). Although the virus was given a number of different names, these were all dropped in 1986 in favour of Human Immunodeficiency Virus (HIV).

HIV is believed to be a lentivirus which is a subfamily of human retroviruses (Levy *et al*, 1985). Two main variants of the virus have been identified and are designated HIV-1 and HIV-2. It is estimated that 5 - 10 million people are infected with HIV-1 worldwide which will lead to nearly 1 million cases of AIDS developing within the next few years (Chin *et al*, 1990). HIV-2 is virtually endemic in certain West African countries (Clavel *et al*, 1987) although isolated cases of HIV-2 infection have been reported in the USA, Brazil and Central Africa (CDC, 1989; Cortes *et al*, 1989). In West African countries the prevalence of HIV-2 in the same high risk groups that are at risk for HIV-1 worldwide, suggest that these viruses share similar modes of transmission. Indeed case reports of co-infection with both variants have been reported in the literature (Rey *et al*, 1987). The abbreviation HIV used in the remainder of this thesis will refer to HIV-1.

1.8.1.1 Transmission

HIV has three main modes of transmission: sexual, parenteral and vertical. Sexual transmission includes homosexual, bisexual or heterosexual contact. Parenteral transmission includes injection with contaminated needles and syringes, and transfusion with infected blood or blood products. Vertical transmission may occur transplacentally, intrapartum and via infected breast milk (Oxtoby, 1988; Falloon *et al*, 1989; Wofsy, 1990).

1.8.1.2 Epidemiology

There are three patterns of HIV infection worldwide that are thought to have developed as a result of global differences in the frequency of the various modes of transmission and the rate of introduction of the virus to different areas. (From WHO after Greenspan *et al*, 1990b). The first pattern affects mainly Western countries including the USA, Western Europe, Australia and New Zealand. In these countries homosexual and bisexual men, and intravenous drug abusers (IVDA) have been the main affected groups, however recent years have seen a decline in the rate of infection of the homosexual population and a rise in both heterosexual and perinatal transmission of the virus. The second pattern affects Africa and South America, where both sexes are infected to a similar degree with heterosexual and perinatal transmission being the most common. The third pattern affects North Africa, Eastern Europe and Asia. In these areas only a small number of AIDS cases have been reported and this is thought to be due to the relatively late introduction of HIV into these areas. Recently a rapid spread of HIV infection has been documented among female prostitutes and IVDA in some of these regions.

1.8.1.3 Natural history of HIV infection

The natural history of HIV infection has been studied mainly in homosexual men and haemophiliacs, because these were the cohorts originally infected, although it is believed that information gathered so far is applicable to the disease as seen in heterosexuals (Moss and Bacchetti, 1989). However, some IVDA in New York appear to die of non-AIDS causes before being diagnosed with AIDS. It is believed that many of these other causes of death may in fact be associated with or exacerbated by HIV infection and that current definitions are thought to underestimate the progression to AIDS in this group (Moss and Bacchetti, 1989).

1.8.1.3.1 Primary infection with HIV

If exposure to HIV results in infection, seroconversion occurs between two to six weeks later and may be associated with an acute clinical illness experienced by 50-90% of adults (Tindall *et al*, 1988). The symptoms of the primary infection with HIV have been described as resembling a mononucleosis-like infection and include; erythematous maculopapular rashes, fever, sweats, malaise, myalgia, arthralgia, headache, photophobia, diarrhoea, sore throat, lymphadenopathy and neurological manifestations including aseptic meningitis (Cooper *et al*, 1985). The illness is characterised by sudden onset and may last from three days to several weeks. The condition is self limiting and the majority of patients become asymptomatic after a few weeks with persistent antibodies normally developing within three months (Moss and Bacchetti, 1989). Long-lasting symptomatic primary HIV infection has been reported to be strongly correlated with subsequent risk of rapid progression to AIDS (Pedersen *et al*, 1989).

1.8.1.3.2 Persistent generalised lymphadenopathy

In a number of patients who experience acute seroconversion illness, generalised lymphadenopathy, established at primary infection, persists after remission of other symptoms. This persistent generalised lymphadenopathy (PGL) can also develop in some patients who were asymptomatic at seroconversion and typically involves two or more extrainguinal sites (Tindall *et al,* 1988).

1.8.1.3.3 AIDS-related complex

There may then follow a period of many years after seroconversion where the infection appears to be in a latent state and the patient is reasonably healthy. However asymptomatic patients and those with PGL can progress to a range of clinical conditions reflecting increased immunosuppression collectively referred to as AIDS-related complex (ARC) (Seligmann *et al,* 1987). These symptoms include multidermatomal herpes zoster (Melbye *et al,* 1987), oral candidiasis (Moss, 1988), oral hairy leukoplakia (Greenspan *et al,* 1987), and lymphoma-like constitutional symptoms of sustained weight loss, fatigue, night sweats and persistent diarrhoea (Moss *et al,* 1988).

1.8.1.3.4 AIDS

Full-blown fatal AIDS, characterised by opportunistic infections, Kaposi's sarcoma or non-Hogkins lymphoma, can develop in previously asymptomatic patients, PGL or ARC patients. It is unusual for patients to survive more than five years after contracting full blown AIDS (Volberding and McCutchan, 1989). It has been estimated that the average time from infection to AIDS is seven to ten years (Moss and Bacchetti, 1989). Neurological dysfunction has been reported in up to 50% of patients in the terminal

phases of AIDS, but may present at any time during the course of the disease (Wiley and Nelson, 1990). Encephalopathy, myelopathy and peripheral neuropathy have been reported in HIV seropositive patients, however the mechanisms of CNS damage remain poorly understood (De Girolami *et al*, 1990; Wiley and Nelson, 1990).

1.8.1.4 Classification of HIV infection

There are two commonly used classification systems for staging HIV infection. The CDC system was initially proposed in 1986 and classifies manifestations of HIV infection into four mutually exclusive groups (Table 1). This classification system was amended in 1987 and revised in 1992 to redefine asymptomatic HIV seropositive patients and ARC patients with $CD4^+$ T-cell counts of less than 200 mm^3 as having AIDS (CDC, 1986, 1987, 1992). This system applies only to patients diagnosed with HIV infection (CDC, after Greenspan *et al*, 1990a). The Walter Reed classification system is based on a variety of clinical and laboratory markers of immunosuppression (Table 2). This system relies on $CD4^+$ T-cell count and function to stage HIV disease (Redfield *et al*, 1986; Redfield and Burke, 1988).

Table 1 CDC Classification of HIV-associated Disease

Group I		Acute infection
Group II		Asymptomatic infection
Group III		Persistent generalized lymphadenopathy
Group IV		Other disease
	Subgroup A	Constitutional disease
	Subgroup B	Neurologic disease
	Subgroup C	Secondary infectious diseases
	Category C-1	infectious diseases listed in the CDC surveillance definition for AIDS
	Category C-2	other specified secondary infectious diseases
	Subgroup D	Secondary cancers
	Subgroup E	Other conditions

From CDC (1986) after Greenspan *et al* (1990).

Table 2 Walter Reed Classification System

WR Stage	HIV	C.L.	$CD4^+/mm^3$	Delayed hypersensitivity skin test	Thrush	Opportunistic infections
0	-	absent	> 400	normal	absent	absent
1	+	absent	> 400	normal	absent	absent
2	+	present	> 400	normal	absent	absent
3	+	varies	< 400	normal	absent	absent
4	+	varies	< 400	partial anergy	absent	absent
5	+	varies	< 400	complete anergy	present	absent
6	+	varies	< 400	partial or complete anergy	absent/ present	present

WR Stage Walter Reed Stage

HIV antigen or antibody

C.L. chronic lymphadenopathy

From Redfield *et al* (1986) after Greenspan *et al* (1990)

1.8.1.5 Structure of HIV

The basic structure of HIV includes a central core consisting of two identical strands of viral RNA intimately associated with enzymes known as reverse transcriptase (Figure 1). Surrounding these elements are two protein layers, a core shell of protein, p24, and an outer layer of another protein, p17, that lies immediately below the viral envelope. The envelope consists of a lipid bilayer that is traversed by a transmembrane protein, gp41. This protein is associated with the external envelope protein, gp120, and gives the virus the appearance of small spikes or protrusions under electron microscopy. The complete virion is estimated to be 100 nm in diameter. (Evans and Levy, 1989).

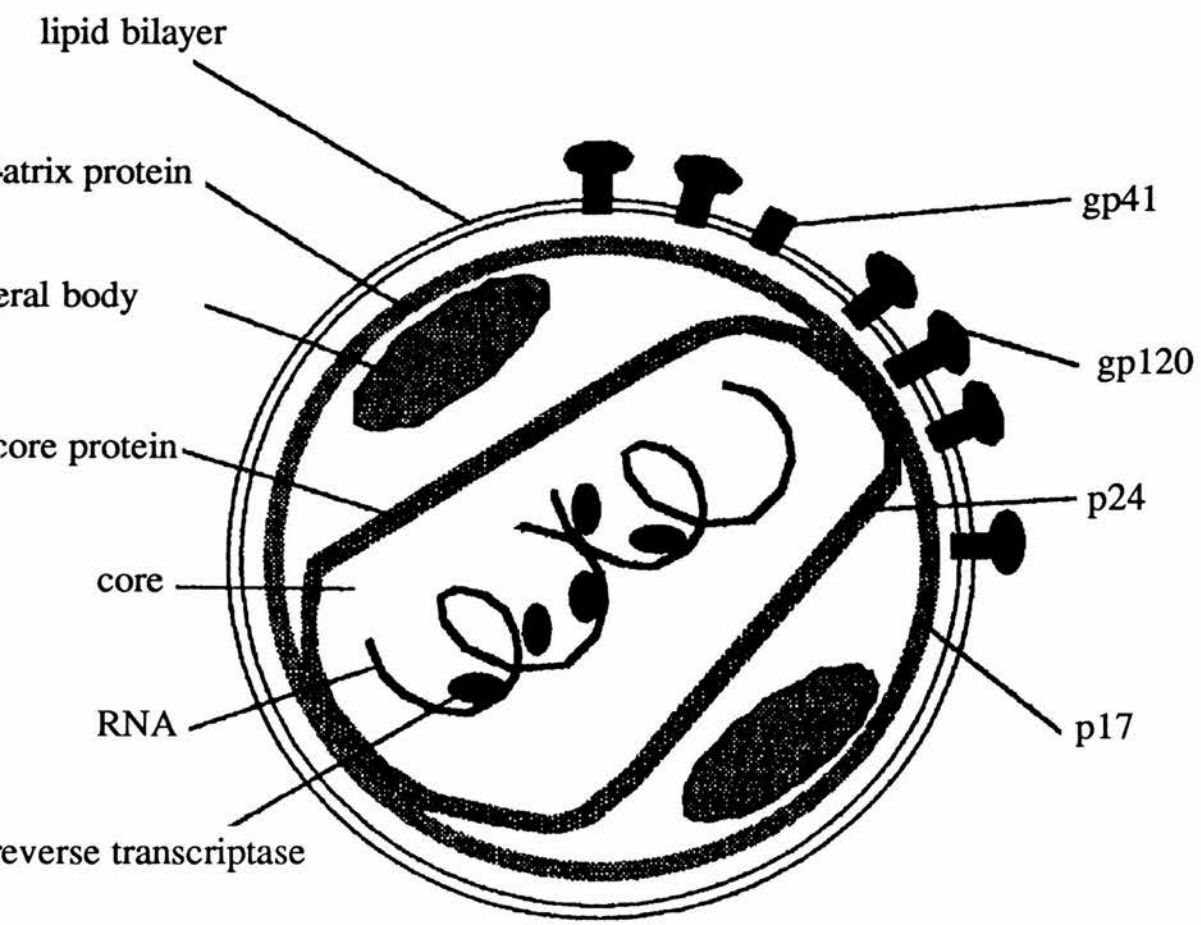


Figure 1 Structure of the Human Immunodeficiency Virus (after Levy, 1990)

1.8.1.6 Life cycle of HIV

1.8.1.6.1 Attachment, fusion and entry

In vitro, the virus displays a tropism for host cells that express the CD4 antigen on their surface, so it is thought that once inside the host the viral envelope protein, gp120 attaches to the surface of cells that display the CD4 antigen e.g. helper T-lymphocytes (Evans and Levy, 1989). Fusion of the virus to the cell surface is thought to be aided by binding of the transmembrane protein, gp41, and a specific fusion receptor on the cell surface (Levy, 1990).

Although clinical disease progression is related to HIV infection and depletion of $CD4^+$ T-lymphocytes, expression of the CD4 antigen is not restricted to these cells and can be expressed to varying degrees on a variety of cell types, including monocytes and macrophages, Langerhans-dendritic cells, follicular dendritic cells of germinal centres, B-lymphocytes transformed by Epstein Barr Virus, glial cells and neurones. All of these cells have been reported to support viral replication (Levy, 1990). However, there may be alternative modes of infection involving cells that do not have CD4 on their surface. *In vitro* infection of brain astrocyte lines, human fibroblasts, and endothelial and epithelial cells of seropositive patients with HIV has been demonstrated (Wiley *et al*, 1986; Nelson *et al*, 1988). Antibody-mediated enhanced infection, either complement-dependent or complement-independent, has also been proposed. This mechanism would probably involve Fc and complement receptors and could take place in peripheral blood macrophages as well as T cells and established T cell lines (Robinson *et al*, 1988).

1.8.1.6.2 Replication

Once inside the cell the reverse transcriptase produces a DNA copy of the viral genome that is integrated into host chromosomal DNA in a double stranded circular form and exists as proviral DNA (Evans and Levy, 1989). The infected cell can remain in this latent state for long periods, producing little or no viral RNA or proteins, and so can escape the host immune system. When active virus production begins the proviral DNA is transcribed into viral RNA and messenger RNA, coding for structural proteins and cell associated regulatory proteins (Levy, 1990). Assembly of progeny virions is thought to occur in the cytoplasm and at the cell membrane. The virus can then be spread by budding from the cell surface or by the process of fusion of an infected cell with uninfected cells (Evans and Levy, 1989).

1.8.1.7 Serological events of infection

An HIV antigenaemia follows infection and specific antibodies to major structural proteins of HIV can be detected within a few weeks of the acute illness or 2 - 6 weeks after initial exposure (Allain *et al,* 1986). HIV and p24 antigen become increasingly difficult to isolate from serum soon after seroconversion and antibodies to core (p24) and envelope proteins (gp41) appear (Pedersen *et al*, 1987). Although antibodies produced by the host are not able to inactivate the virus, the infection appears to enter a latent phase. These events coincide with resolution of the acute illness and the patient may remain asymptomatic and reasonably healthy for several years (Tindall, 1990). Eventually, however, p24 antigen reappears in the blood as antibodies to p24 begin to disappear. These events have been associated with a deterioration of the clinical condition of the patient with the occurrence of opportunistic infections and a progression to AIDS (Pedersen *et al*, 1987). Production of antibodies to envelope

proteins generally remains consistent throughout the course of the disease (Allain *et al*, 1987).

1.8.2 HIV Infection and Host Defences

Infection with HIV can lead to a range of abnormalities of inflammatory and immune systems, some of which may be present without evidence of clinical illness. As the patient progresses to full-blown AIDS, however, these abnormalities tend to become more extreme (Sattentau, 1989).

1.8.2.1 T-lymphocytes

The alteration of $CD4^+$ T helper/inducer and $CD8^+$ T suppressor lymphocyte subsets is one of the most consistent observations in patients infected with the virus. An inverted $CD4^+$: $CD8^+$ ratio results from markedly reduced $CD4^+$ T cell numbers and stable or raised number of $CD8^+$ T cells (Fauci *et al*, 1984). Soon after primary infection $CD4^+$ T cell numbers fall from normal levels of more than $500/mm^3$ blood in the majority of patients over the course of the disease. Most AIDS defining opportunistic infections occur in patients with fewer than $200/mm^3$ $CD4^+$ T cells (Volberding and McCutchan, 1989). In addition to a reduction in numbers of $CD4^+$ T cells, HIV infection also results in abnormalities of $CD4^+$ T cell function. As these cells have a central role in the immune system this can lead to a range of defects including decreased blast transformation of T and B lymphocytes to mitogens and antigens; diminished cytotoxic T cell response and depressed helper function for B cell immunoglobulin production (Bowen *et al*, 1985; Seligmann *et al*, 1987).

1.8.2.2 Cytokines

HIV infection is also thought to affect cytokine production, which itself is intimately associated with the function of T lymphocytes and their modulation of the immune response. A reduction in IL-2 production and activity has been documented in HIV infected patients. (Shearer *et al*, 1986). This phenomenon may be partly the result of decreased $CD4^+$ T cell numbers, as IL-2 is mainly produced by these cells (Sattentau, 1989). Increased levels of TNF found in serum of HIV infected patients are believed to stimulate HIV replication (Sattentau, 1989).

1.8.2.3 B-lymphocytes

It has been shown that B-lymphocytes are polyclonally activated in patients with HIV (Seligmann, 1987). This results in elevated serum immunoglobulin levels of all isotypes, circulating immune complexes, autoimmune disease and an inability of B cells to respond normally to antigenic stimulation (Sattentau, 1989). Polyclonal activation of B cells could be the result of either direct infection of these cells with HIV or the mitogenic response of B cells to the virus.

1.8.2.4 Natural killer cells (NK)

The lack of IL-2 is thought to be responsible for the reduction in lysis by natural killer cells observed in some HIV seropositive patients (Sattentau, 1989).

1.8.2.5 Monocytes and macrophages

Chemotaxis, Fc-dependant phagocytosis, intracellular killing, nonspecific cytotoxicity and hydrogen peroxide release have all been shown to be abnormal in

monocytes/macrophages *in vitro* (Smith *et al,* 1984; Pinching *et al*, 1983) Antigen presentation by these cells is also thought to be impaired. Although some of these defects are the direct result of lack of stimulation by $CD4^+$ T-lymphocytes, direct infection with HIV also impairs their function. (Sattentau, 1989)

1.8.2.6 Polymorphonuclear leucocytes

Kinne and Gupta (1989) reported deficient PMNL-mediated antibody-dependent cellular cytotoxicity in HIV seropositive patients and suggested this as the cause of increased predisposition to bacterial infections observed in some patients (Polsky *et al,* 1986). However, elevated phagocytosis and oxidative burst in PMNL has been described in some HIV seropositive patients (Ryder *et al*, 1988), whereas serum from homosexual HIV seropositive patients with HIV-associated periodontitis was found to cause impairment of PMNL killing (Winkler and Hammerle, 1991). Interestingly, Lazzarin *et al* (1986) found defective migration of PMNL from non-IVDA HIV seropositive patients, but defective phagocytosis and killing in PMNL from a group of IVDA HIV seropositive patients.

1.8.3 Cofactors and Predictors of Disease Progression

In order to explain the apparent differences in disease progression between individuals infected with HIV there have been attempts to identify possible cofactors (Polk *et al*, 1987). Several clinical markers have been proposed as predictors of disease progression, but they differ widely in predictive power (Greenspan *et al*, 1987; Moss, 1988: Moss *et al*, 1988; Fahey *et al,* 1990). Although Herpes zoster, oral candidiasis and oral hairy leukoplakia were found to be mid-range predictors of progression to full-blown AIDS i.e. unlikely to occur until 3 - 5 years after primary infection with HIV, they varied widely in predictive power. However, lymphoma-like constitutional

symptoms were regarded as highly predictive for imminent progression to AIDS (Moss and Bacchetti, 1989).

A number of laboratory markers have also been shown to predict disease progression in HIV seropositive asymptomatic patients (Moss *et al,* 1988). In a comparison of several cellular and serological markers of HIV infection, $CD4^+$ T lymphocytes expressed as an absolute number, percentage of lymphocytes, or a ratio of $CD4^+$ to $CD8^+$ T cells was found to be the best single predictor of progression to AIDS. However, increased serum levels of neopterin, a metabolite produced by stimulated macrophages, and β_2-microglobulin, a non-covalently linked protein component of the major histocompatibility complex, were found to have almost as much predictive power. Levels of IgA, IL-2 and p24 antigen were found to have less predictive value (Fahey *et al*, 1990). These authors, along with Moss and Bacchetti (1988), concluded that the progression to AIDS was most accurately predicted by the level of $CD4^+$ T cells in combination with serum levels of either neopterin or β_2-microglobulin.

1.8.4 Periodontal Disease and HIV infection

1.8.4.1 History

Winkler *et al* (1986) reported an aggressive periodontitis and an atypical gingivitis occurring in patients infected with HIV. These authors described the appearance of a rapidly progressive periodontitis, named AIDS-virus associated periodontitis (AVAP) in 8 HIV seropositive, 9 ARC and 21 AIDS patients (Winkler *et al,* 1987). AVAP was originally described as having a similar clinical appearance to ANUG. However, *spirochaetes* were absent from these lesions (Winkler *et al*, 1986), and rapid destruction of periodontal attachment and bone were common clinical features of the lesion (Winkler *et al*, 1987). Other clinical features included rapid onset, interproximal soft

tissue necrosis, ulceration and cratering, marked oedema and intense erythema of the attached and marginal gingivae. Patients commonly complained of severe pain and spontaneous nocturnal bleeding from the gingivae (Winkler *et al*, 1986, 1987, 1988). Response to conventional treatment was noted to be slow and some lesions remained unresolved after several weeks (Winkler *et al*, 1986).

The atypical gingivitis was reported in 24 homosexual men previously unaware of exposure to HIV (Winkler *et al*, 1986). The distinguishing clinical features of this lesion were a red linear-type border extending from the attached gingiva and red petechia-like patches occurring on the attached and unattached gingiva (Winkler *et al*, 1986, 1987). This lesion was also found to be unresponsive to conventional oral hygiene procedures. Subsequent HIV antibody testing of these patients found all but one to be HIV seropositive. This finding prompted speculation by the authors that the atypical gingivitis may be a early intra-oral expression of HIV infection (Winkler *et al*, 1987).

Both lesions were frequently found adjacent to apparently clinically healthy gingiva and periodontium. The AVAP and the atypical gingivitis were subsequently renamed HIV-associated periodontitis (HIV-P) and HIV-associated gingivitis (HIV-G) respectively (Winkler *et al*, 1988).

In 1990, a further disease entity involving the periodontal tissues of HIV seropositive subjects was observed and termed necrotising stomatitis (Williams *et al*, 1990). This severe condition has been described as originating from the periodontium and can result in widespread and rapid destruction of the periodontium, as well as adjacent hard and soft tissues. Several case reports of this lesion are have been recorded (Williams *et al*, 1990; SanGiancomo *et al*, 1990; Felix *et al*, 1991).

1.8.4.2 Classification

There have been several attempts to classify periodontal diseases occurring in HIV seropositive patients (Winkler *et al*, 1989; Axéll *et al*, 1991; Challacombe *et al*, 1991; Smith *et al*, 1993).

The classification systems proposed by Winkler *et al* (1989, 1992) have several disadvantages. For example there is no provision for non-HIV periodontal diseases in HIV seropositive patients. Inappropriate nomenclature suggests involvement of HIV in the aetiology of periodontal diseases and creates the potential for breaches in confidentiality (Smith *et al*, 1993). Recently, Masouredis *et al* (1992) proposed two sets of clinical criteria for the diagnosis of HIV-G and HIV-P based on whether or not probing was included in the assessment. However, the system described by Winkler *et al* (1988, 1992) has been the most frequently used and, in the absence of a universally agreed classification system, was adopted for use in the present study.

1.8.4.2.1 HIV-associated gingivitis

HIV-G has been described as a profound erythema of the gingivae that in the majority of cases presents as a red linear band extending two to three millimetres apically from the gingival margin. Punctate and diffuse erythema may be accompanying features of this condition and can involve the entire attached gingivae extending from the gingival margin to the alveolar mucosa. Lesions generally affect the whole mouth and are evenly distributed throughout all quadrants. It has been reported that 15% of HIV-G sites show bleeding on probing with 11% bleeding spontaneously (Winkler *et al*, 1988, 1992).

1.8.4.2.2 HIV-associated periodontitis

HIV-P includes all the clinical features of HIV-G in addition to soft tissue necrosis with accompanying gingival bleeding and rapid destruction of periodontal attachment. Severe pain is a common complaint being described as a 'deep aching' pain localised to the 'jaw bone' which may herald the onset of an acute phase of the disease before other clinical signs. Bleeding on probing is a consistent feature with 50% of lesions exhibiting spontaneous bleeding that may result in nocturnal bleeding with patients complaining of blood clots on pillows or in their mouths on awakening (Winkler *et al*, 1986). The rapid progression of soft tissue necrosis can result in exposure of crestal and interseptal alveolar bone which may sequestrate. HIV-P is usually a localised lesion often resulting in the appearance of a severely affected site adjacent to sites with signs of HIV-G or relatively normal tissue occurring round the same tooth (Winkler *et al*, 1988, 1992).

1.8.4.2.3 Necrotising stomatitis

Case reports of HIV seropositive patients with necrotising stomatitis have appeared recently in the literature (Williams *et al*, 1990; SanGiancomo *et al*, 1990; Felix *et al*, 1991). This lesion is characterised by rapid destruction of affected tissues resulting from ulceration and subsequent necrosis. Originating from the gingivae, the lesion extends into adjacent tissues including the oral mucosa and underlying bone, and can result in denudation and sequestration of bone. Necrosis of confluent tissues is a distinguishing feature of necrotising stomatitis (Winkler and Robertson, 1992).

1.8.4.2.4 Acute necrotising ulcerative gingivitis

Cases of ANUG occurring in HIV seropositive patients have been documented (Pindborg, 1986; Pindborg and Holmstrupp, 1987) and also in homosexual males whose

HIV status was unknown (Dennison *et al*, 1985). The clinical features of ANUG in HIV seropositive patients are essentially indistinguishable from the disease occurring in HIV seronegative subjects, however it may be unusual in extent, severity or age distribution (SchØidt and Pindborg, 1987; Smith *et al*, 1993).

Although HIV-P was initially described as being similar to ANUG (Winkler *et al*, 1986, 1987) clinical and microbiological features can help distinguish between the two lesions (Winkler and Murray, 1987; Winkler *et al*, 1986, Murray *et al*, 1988). For example, HIV-P is associated with rapid destruction of both soft and hard tissues, whereas ANUG only results in loss of bone after multiple episodes over several years (Winkler and Murray, 1987). Furthermore, the levels of *spirochaetes* present in lesions of HIV-P are variable and the microbiota of these lesions is not consistent with that associated with ANUG (Murray *et al*, 1988).

1.8.4.2.5 Conventional periodontal diseases

Non-specific gingivitis and adult periodontitis are thought to occur in HIV-seropositive patients in the same way as in HIV-seronegative patients and may be established long before HIV infection. Furthermore, it is suspected that rare periodontoses, such as prepubertal periodontitis and localised juvenile periodontitis can also occur in HIV seropositive patients (Smith *et al*, 1993).

1.8.4.3 Prevalence

The prevalence of periodontal disease in HIV seropositive patients has been reported in numerous studies of the oral manifestations of HIV infection and AIDS and in studies concerned with HIV-associated periodontal diseases (Silvermann *et al*, 1986; Phelan *et al*, 1987; Roberts *et al*, 1988; Schulten *et al*, 1989; Porter *et al*, 1989; Tukutuku *et al*,

1990; Barone *et al*, 1990; Laskaris *et al*, 1992; Gillespie and Mariño, 1993; Murray *et al*, 1991a; Friedman *et al*, 1991; Swango *et al*, 1991; Masouredis *et al*, 1992).

However, results from studies of the oral manifestations of HIV infection vary with respect to prevalence of gingivitis, from 1% to 66% of subjects, and prevalence of periodontitis which varied from 0% to 91% of subjects studied (Phelan *et al*, 1987; Tukutuku *et al*, 1990; Roberts *et al*, 1988; Barone *et al*, 1990; Klein *et al*, 1991). Prevalence of HIV-associated periodontal diseases, from periodontal research papers, range from 0% to 50% of subjects affected by HIV-G and from 0% to 22% of subjects with HIV-P (Apaiza *et al*, 1991; Freidman *et al*, 1991; Swango *et al*, 1991; Murray *et al*, 1991a: Masouredis *et al*, 1992).

Variations in the prevalence of periodontal diseases reported by these studies have been attributed to geographic variation (Gillespie and Mariño, 1993), selective sampling, improper diagnostic criteria and poor study design (Robinson, 1993). Standardised diagnostic criteria will have to be established before accurate prevalence rates of periodontal diseases in HIV seropositive patients can be determined (Robinson, 1993; Smith *et al*, 1993).

1.8.4.4 Histology

Few reports on the histology of HIV-associated periodontal disease have been documented in the literature. One report was concerned with the histological features of HIV-G from a single biopsy of an HIV seropositive patient complaining of bleeding gums. The main features were of gingival tissue engorged with blood vessels, but the absence of any inflammatory infiltrate (Glick *et al*, 1990). Steidley *et al* (1992) attempted to characterise T cell subpopulations in gingival tissue from eight HIV seropositive patients and compare to six HIV seronegative controls. The gingival tissue

for all patients was collected during routine periodontal surgery as part of the treatment for periodontal disease. Using monoclonal antibodies to $CD4^+$ and $CD8^+$ T cells they found that the gingival tissue from the HIV seropositive patients exhibited a complete absence of T cells in all but one patient. All tissue taken from the controls had these cells present (Steidley *et al*, 1992).

1.8.4.5 Microbiology

A number of studies have investigated the microbiology of subgingival plaque samples from HIV seropositive patients using a variety of sampling and identification techniques (Murray *et al*, 1988, 1989, 1991b; Zambon *et al*, 1990; Lucht *et al*, 1991; Gornitsky *et al*, 1991; Rams *et al*, 1991; Moore *et al*, 1993). Some of these studies have characterised the subgingival microbiota of HIV seropositive patients and have investigated HIV-associated periodontal disease in only a few patients (Zambon *et al*, 1990; Lucht *et al*, 1991). Others have attempted to characterise the microbiota specifically associated with HIV-G and HIV-P (Murray *et al*, 1988, 1989, 1991b; Gornitsky *et al*, 1991; Rams *et al*, 1991).

The subgingival microflora of HIV seropositive patients would appear to be qualitatively similar to that of HIV seronegative patients. Zambon *et al* (1990), using the predominant cultivable microbiota technique and immunofluorescence microscopy, found many of the Gram-negative anaerobic microorganisms associated with conventional periodontitis. Similarly, Lucht *et al* (1991), also using predominant cultivable techniques, detected no significant differences between the subgingival microflora found in saliva or gingival crevice in 10 HIV seronegative, 10 seropositive, 10 ARC and 10 AIDS patients. A recent study by Moore *et al* (1993) found that the only difference in the subgingival microbiota between HIV seropositive and

seronegative patients, both with adult periodontitis, was a statistically significantly elevated levels of *Mycoplasma salivarium* in HIV seropositive subjects.

Murray *et al* (1988, 1989, 1991b) have used a variety of techniques including predominant cultivable microbiota, indirect immunofluorescence, indirect ELISA and DNA probes to determine the subgingival microbiota associated with HIV-G and HIV-P. These workers have found the microbiota of both of these lesions to contain similar subgingival species that are found in conventional periodontitis including *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *F. nucleatum*, *C. rectus*, *E. corrodens* . However, using DNA probes *C. rectus* was found in higher proportions in HIV-P sites than in HIV-G sites (Murray *et al*, 1991).

Using the predominant cultivable microflora technique Rams *et al* (1991) compared the subgingival flora for 3 clinically healthy and 3 HIV-P sites in 14 HIV seropositive patients. The HIV-P sites harboured Gram-negative anaerobic species similar to those found in conventional periodontitis including *A. actinomycetemcomitans*, *P. intermedia* and *C. rectus* and concluded that the microbiota of HIV-P sites was similar to that found in progressing periodontal lesions in systemically healthy adults (Rams *et al*, 1991). Gornitsky *et al* (1991), reported no differences in the microbiological profile between HIV-P sites and periodontitis sites from healthy HIV seronegative patients, using darkfield and immunofluorescent microscopy techniques directed towards a limited number of organisms.

Interestingly, a number of studies investigating subgingival plaque samples from HIV seropositive patients have reported finding microorganisms not usually associated with the subgingival microflora and others at higher levels than previously detected in healthy adults with severe periodontitis (Murray *et al*, 1988; Slots *et al*, 1988; Zambon *et al*, 1990; Rams *et al*, 1991). Murray *et al* (1988) found a significantly elevated

percentage of sites harbouring *C. albicans* in HIV-P and HIV-G lesions than in healthy sites from HIV seropositive subjects or sites from seronegative controls. Both Rams *et al* (1991) and Zambon *et al* (1990) reported recovering microorganisms not normally associated with adult periodontitis from subgingival plaque samples from HIV seropositive patients including, *Bacteroides fragilis*, *Fusobacterium necrophorum, Fusobacterium varium, Eubacterium aerofaciens, Candida albicans, Pseudomonas aeruginosa, Klebsiella oxytoca, Enterobacter cloacae, Enterobacter agglomerans*, uncommon *Clostridium* species and enterococci. Felix *et al* (1991) reported high levels of *Enterobacter cloacae, Enterococcus* and coliforms from a lesion of necrotising stomatitis.

Schmidt-Westhausen *et al* (1990), in a retrospective study of routine oral swabs, detected *Enterobacteriaceae* in 5% of HIV seropositive patients and in 4.8% of a control group of HIV seronegative patients. However, closer inspection of the control group revealed that of those with *Enterobacteriaceae* all were predisposed to opportunistic infections as they were either diabetics or had undergone irradiation.

In summary, using a variety of sampling and identification techniques the majority of studies analysing the subgingival microbiota of HIV seropositive and HIV-associated periodontal lesions are in agreement. The subgingival microbiota of HIV seropositive patients harbours a similar range of organisms found in the subgingival microbiota of HIV seronegative healthy adults (Lucht *et al*, 1991; Zambon *et al*, 1990; Moore *et al*, 1993). The range of organisms isolated from HIV-associated periodontal lesions is similar to that found in conventional adult periodontitis (Murray *et al*, 1988, 1989, 1991b; Rams *et al*, 1991; Gornitsky *et al*, 1991). However, a number of studies have detected microorganisms not previously regarded as common subgingival colonisers, in low or moderate levels, from healthy and diseased sites in HIV seropositive patients (Murray *et al*, 1988; Zambon *et al*, 1990; Rams *et al*, 1991).

1.8.4.6 Immunology

The most consistent feature of infection with HIV is the progressive loss and deterioration in function of $CD4^+$ T cells, combined initially with a relative rise in $CD8^+$ T cells. As a result of this there has been considerable interest in the levels of $CD4^+$ and $CD8^+$ T cells in peripheral blood and local tissue of HIV seropositive patients with HIV-associated periodontal diseases (Winkler *et al*, 1988; Lucht *et al*, 1991; Steidley *et al*, 1992; Barr *et al*, 1992). Winkler *et al* (1988) found in patients with HIV-G the $CD4^+$: $CD8^+$ ratio was at the low end of the normal range, but patients with HIV-P had significantly lower $CD4^+$: $CD8^+$ ratios than normal. Steidley *et al* (1992) reported that low $CD4^+$ T cell counts in peripheral blood correlated well with severity of HIV-P observed. In another study, significantly more attachment loss was observed in HIV seropositive patients with $CD4^+$ T cell counts of less than $200/mm^3$ blood compared to HIV seropositive patients with $CD4^+$ T cell counts of greater than $200/mm^3$ blood (Barr *et al*, 1992).

To date only one study has reported levels of $CD4^+$ and $CD8^+$ T cells in gingival tissue taken from HIV seropositive patients with HIV-P. Steidley *et al* (1992) reported that no $CD4^+$ or $CD8^+$ T lymphocytes observed in gingival tissue from all but one of 8 HIV seropositive patients with HIV-P. The HIV seropositive patient with some $CD4^+$ and $CD8^+$ T cells present had near normal levels of serum $CD4^+$ T cells.

1.8.4.7 Pathogenesis

Winkler *et al* (1988) demonstrated $CD4^+$: $CD8^+$ ratios within the low normal range in patients with HIV-associated gingivitis, but significantly below normal ratios in patients with HIV-associated periodontitis. This group of patients had elevated serum antibodies to *P. gingivalis, P. intermedia, F. nucleatum, E. corrodens* and

A. actinomycetemcomitans relative to HIV-negative heterosexual and homosexual controls. These authors proposed that the pathogenesis of HIV-associated periodontal diseases was the result of the compromised immune system of the HIV infected patient allowing shifts in virulence potential of the oral microbiota. Therefore tissue destruction would result from an overgrowth of specific pathogens during periods when the tissue defence mechanisms were compromised. This same group also reported altered PMNL function in HIV seropositive patients, including increased phagocytosis and oxidative burst, compared to HIV seronegative controls (Ryder *et al,* 1988). They proposed that hyper-responsive PMNL, primed by bacteremia of oral origin and in the absence of normal functioning lymphocytes and macrophages, could contribute to the severe periodontal disease observed in some HIV seropositive subjects. Lucht *et al* (1991) also found increasingly severe periodontal disease with progressing HIV infection, exemplified by an inverse relationship between peripheral blood $CD4^+$ T-lymphocytes and both gingivitis and pocket depth. However, these authors concluded that non-specific accumulation of dental plaque or potential specific pathogens were unlikely to play a major role in the development of periodontal diseases in HIV seropositive patients, rather that the severity of periodontal disease was correlated with immunosuppression and progression of HIV infection. Steidley *et al* (1992) proposed that a reduction in $CD4^+$ T-lymphocytes in ginigval tissue could contribute to the development of the severe periodontal diseases seen in HIV seropositive patients. Robinson (1992) in a review of HIV-asociated periodontal diseases, concluded that several explanations for the aetiology of periodontal diseases in HIV seropositive patients were possible. They could either be the result of infection by the subgingival species currently associated with conventional periodontitis whose virulence is increased with the diminished immune response in HIV or, unusual species found colonising the subgingival area in HIV seropositive subjects, found by some workers, contributing to the disease.

In summary, similarities in clinical appearance and microbiota associated with HIV-G and HIV-P have prompted speculation that HIV-G may be a precusor to HIV-P and represents an earlier stage of the same disease process (Murray *et al*, 1989; Winkler and Robertson, 1992). Furthermore, case reports of necrotising stomatitis indicate that this lesion may be a further stage of HIV-P (Williams *et al*, 1990; SanGiancomo *et al*, 1990; Felix *et al*, 1991). Therefore, HIV-associated periodontal diseases may represent a spectrum of the same disease process and that the particular periodontal lesion seen in a patient may depend on the subgingival microbiota that emerges during periods of immunosuppression, which in turn will depend on the degree, duration and type of alteration of the immune response (Winkler and Robertson, 1992).

1.8.4.8 Management

Due to the recognition that HIV-G may be an early stage of HIV-P, prompt and vigorous treatment of HIV-G lesions with aggressive scaling and root planing in addition to 0.12% chlorhexidine mouthwash has been advocated (Grassi *et al*, 1988; Murray *et al*, 1989).

The management of severe periodontal disease in HIV seropositive patients was outlined by Winkler and Robertson (1992). Treatment of HIV-P and necrotising stomatitis should involve removal of plaque and calculus and thorough debridement of necrotic tissue with removal of exposed necrotic bone sequestra. These authors also recommended the use of povidone-iodine irrigation during debridement as it helps to relieve pain and reduce bleeding. Chlorhexidine mouthwash, twice daily, should be started as soon as possible and may be continued throughout long-term maintenance. Immediate follow-up care should involve quadrant-by-quadrant scaling and root planing with removal of necrotic tissue as required in addition to instruction in oral hygiene procedures, with follow-up visits every month. Once the condition is stabilised, recall

visits can be arranged at three monthly intervals. Winkler and Robertson (1992) also suggest that the use of systemic antimicrobials be restricted to the immediate phase of treatment and depend on the rate of destruction of the lesion and systemic manifestations. Metronidazole is the drug of choice with a short course, low dose regimen of 250 mg four times a day for 4 - 5 days advocated. The use of broad-spectrum systemic antimicrobials is not recommended, however, due to the potential for candidiasis to develop. Indeed, these authors advise that patients should be monitored for candidiasis throughout the period of metronidazole therapy and antifungals used if required.

1.9 OBJECTIVES OF STUDY

Previous studies of the subgingival microbiota of HIV seropositive subjects have reported data from only a few sites per subject. However, to determine the association between site-specific tissue breakdown and specific subgingival microorganisms, a large number of sites need to be investigated. The development and validation of whole chromosomal digoxigenin-labelled DNA probes for the rapid identification of a selection of periodontal bacteria (Gunaratnam *et al*, 1992), has allowed the analysis of a larger number of subgingival plaque samples than previously possible with traditional cultural identification techniques. The objectives of this thesis were

1) To establish a procedure for the enumeration of selected periodontal bacteria in a large number of plaque samples using non-isotopic whole chromosomal DNA probes.

2) To compare the prevalence of periodontal disease between HIV seropositive and HIV seronegative subjects in the Edinburgh area.

3) To compare the prevalence of these selected periodontal bacteria between HIV seropositive and HIV seronegative patients.

4) To assess the incidence of HIV-associated periodontal diseases in the HIV seropositive group on a longitudinal basis.

5) To characterise the microbiology associated with these lesions in terms of those species selected for enumeration.

6) To investigate whether periodontal breakdown or any of the clinical or microbiological parameters studied could be used as predictors of HIV disease progression.

Therefore, the objectives of the study comprised of cross-sectional and longitudinal components. Initially, baseline clinical measurements and analysis of subgingival plaque samples allowed an assessment of the clinical and microbiological status in HIV seropositive and HIV seronegative subjects from the Edinburgh area. Longitudinal monitoring of HIV seropositive subjects would provide an indication of the incidence of severe periodontal disease in these patients and give an opportunity to characterise the microbiota associated with these lesions in terms of the periodontal species selected for enumeration. Furthermore, longitudinal data collected from the HIV seropositive subjects would provide information on the relationship between periodontal breakdown and HIV disease progression.

CHAPTER 2

MATERIALS AND METHODS AND PRELIMINARY EXPERIMENTS

2.1 LABORATORY PROCEDURES

All chemicals quoted in Materials and Methods were obtained from Sigma (U.K.) unless otherwise stated.

2.1.1 Bacterial Strains

The following species and strains were chosen for enumeration in subgingival plaque samples; *Actinobacillus actinomycetemcomitans* FDC Y4; *Prevotella intermedia* FDC 581; *Porphyromonas gingivalis* FDC 381; *Bacteroides forsythus* ATCC 33277; *Capnocytophaga ochracea* NCTC 11545; *Veillonella parvula* NCTC 11463; *Campylobacter rectus* NCTC 11489; *Fusobacterium nucleatum* NCTC 10562; *Streptococcus sanguis* NCTC 7863. These species were obtained as lyophilized specimens from the various sources given.

2.1.2 Culture Media

Bacteria above were cultured anaerobically in *Bacteroides* medium cooked meat broth (BM CMB, Holbrook *et al,* 1978), with the exception of *B. forsythus,* and *C. rectus* which were cultured in special broths (Dr Anne Tanner, personal comminucation, Appendix 1).

2.1.3 Solid Media

All species were grown anaerobically on Trypticase soy agar supplemented with 5% horse blood (TSBA) plates, with the exception of *P. gingivalis, B. forsythus,* and

C. rectus, which were grown special solid media (Dr Anne Tanner, personal comminucation, Appendix 1).

2.1.4 Anaerobic Cabinet

A Don Whitley mark III Anaerobic Workstation (Don Whitley Scientific Ltd., Shipley, England.) was used to achieve and maintain an anaerobic enviroment containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide at a temperature of 37°C.

2.1.5 Reduced Transport Fluid

Reduced Transport Fluid (RTF) was proposed by Syed and Loesche (1972) as a suitable means of transporting subgingival anaerobic bacteria (Appendix 1). Freshly prepared RTF was filter-sterilised 24 h prior to use under a vacuum through a cellulose acetate filter (pore size 0.22 mm) in a Nalgene 500 ml filter holder with a 1000 ml receiver (Nalgene, New York, U.S.A.) and left in the anaerobic cabinet overnight.

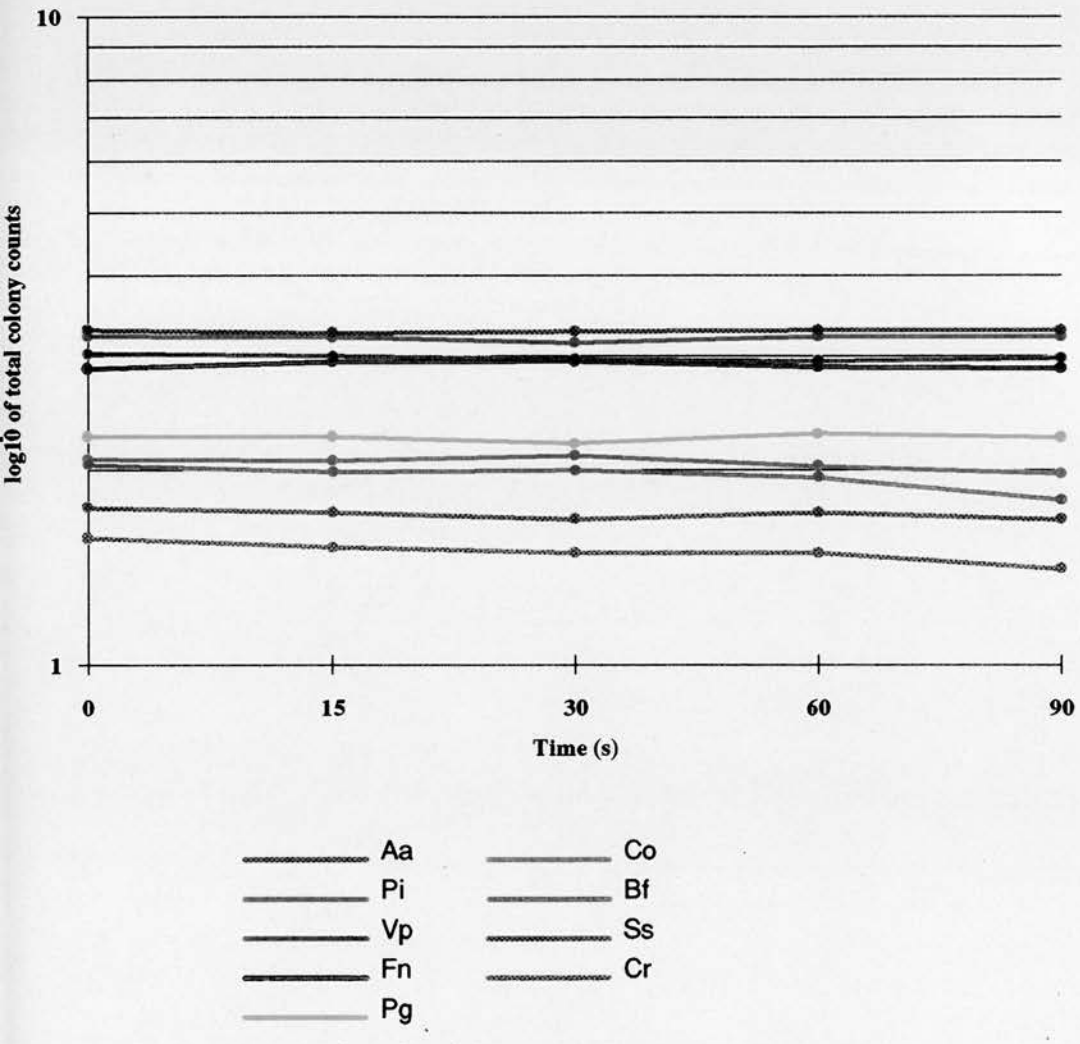
2.1.6 Sonication Experiment

2.1.6.1 Aim

The aim of this experiment was to assess the possible detrimental effect of sonic energy on the nine species listed above, as it was intended to disperse plaque samples in closed bijoux immersed in an ultrasonic waterbath (Weiner *et al*, 1979). Of particular concern were the Gram-negative species, known to be more vulnerable to sonication.

2.1.6.2 Materials and methods

Pure broth cultures of the nine species listed above were anaerobically incubated for two days in the anaerobic cabinet. Aliquots of 0.5 ml of the broths were diluted in 4.5 ml RTF in plastic 7 ml disposable bijoux (Sterilin Medical, U.K.) and sonicated for various times by immersion in the ultrasonic waterbath (Decon FS 100b, Decon Laboratories Ltd, Sussex, England.). After 15s, 30s, 60s and 90s sonication, 100 ml aliquots of the diluted broths were spread onto TSBA plates and anaerobically incubated for 5 - 7 days. A colony counter (Dept. of Medical Microbiology, University of Edinburgh) was used to enumerate the colonies present on the agar plates and assess the effect of sonication on the nine species.



igure 2 Effect of Sonication on Viability of Bacterial Strains Selected for Enumeration

2.1.6.3 Results and Conclusion

The $\log_{10}$ counts for nine strains are plotted against time sonicated in Figure 2. The results of this experiment indicate that these bacterial strains could withstand exposure to sonication in a ultrasonic waterbath for up to 60s without dramatically affecting the total colony counts recovered. It was decided that a plaque sample would be sonicated for 60s to achieve dispersal using this method.

2.1.7 DNA Extraction Methods

A number of DNA extraction methods were used to obtain fragments of whole chromosomal DNA from the nine species based on the rapid DNA extraction method reported by Smith *et al* (1989b).

2.1.7.1 Method A

After anaerobic incubation for 5 - 7 days, growth from the surfaces of 2 - 3 appropriate agar plates of each species were scraped using a disposable plastic loop (Sterilin Medical, U.K.) and resuspended in 567 µl TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA) in sterile 1.5 ml Eppendorf microcentrifuge tubes (Elkay, U.K.). Cells were throughly dispersed by repeated pipetting through a sterile glass Pasteur pipette. Then 30 µl of 10% sodium dodecyl sulphate (SDS) and 3 µl of a 20 mg/ml solution of proteinase K were added. Cell lysis was completed by incubation in a water bath at 37°C for 60 min. After the addition of 150 µl 5 M NaCl to adjust salt concentration, the contents of the Eppendorf were mixed thoroughly, then 85 µl CTAB/NaCl solution (Appendix 1) was added, the reagents mixed again and incubated at 65°C, with occasional mixing, for a further 20 min. An equal volume of chloroform : isoamyl alcohol (24 : 1, v/v) was added, and the contents of the Eppendorf

mixed by shaking vigorously for 5 min to remove cellular proteins and polysaccharides, and then centrifuged for 15 min at 12,000g in a microcentrifuge (Micro Centaur Centrifuge, MSE Scientific Instruments, U.K.). The upper aqueous phase was removed using sterile wide-mouthed Pasteur pipette and transferred to a new sterile Eppendorf tube. An equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v/v. Appendix 1) was added to this aqueous solution and mixed by vigorous shaking for 5 min. The Eppendorfs were centrifuged for a further 12 min and the upper aqueous phase removed by wide-mouthed Pasteur pipette transferred to a new Eppendorf and mixed with 0.6 volumes of isopropanol to precipitate nucleic acids. Following centrifugation for 2 min and removal of the aqueous solution the precipitate was redissolved in 300 µl 10 : 1 TE buffer. From stock solution, 2 µl RNase A was added (Appendix 1) and incubated for 60 min at 50°C. Suspensions were then mixed with 30 µl 3 M sodium acetate, pH 7.6 and re-extracted by adding an equal volume of chloroform : isoamyl alcohol (24 : 1, v/v) as before. Following centrifugation for 6 min, the upper aqueous phase was removed and transferred to a new Eppendorf by sterile wide-mouthed Pasteur pipette. Two volumes of ice cold 95% ethanol (approximately 660 µl) were added and mixed by shaking. The DNA was precipitated by putting the Eppendorf in a freezer at - 20°C for 30 min, and then centrifuged for 5 min. The aqueous solution was removed with a long drawn-out Pasteur pipette and the precipitate washed twice with ice cold 70% ethanol. Following the removal of the last ethanol wash the precipitate was dried in a 37°C incubator for 20 min and dissolved at 37°C overnight in 200 µl 10 : 0.1 TE buffer (10 mM Tris, pH 7.6, 0.1 mM EDTA).

2.1.7.2 Method B: Modified DNA extraction method for Gram-negative species

Confluent growth from 2-3 agar plates was scraped into a sterile 1.9 ml Eppendorf microcentrifuge tube containing 310 µl of 50 mM Tris HCl, pH 8.0, 20 mM EDTA (HTE). Each pellet was well dispersed by repeated passage through a sterile glass

Pasteur pipette to achieve a homogeneous suspension. Lysis was achieved by adding 350 µl of a 2% solution of sarcosyl in HTE and vortexing briefly (Whirlimixer, Fisons Scientific Equipment, U.K.) before adding 5 µl RNase A (Appendix 1) and incubating for 15 min at 37°C. Lysis was completed by adding 35 µl pronase and incubating for 90 min at 50°C. Salt concentration was adjusted by adding 175 µl of 5 M sodium chloride and mixing the contents of the Eppendorf by vigorous shaking. Following the addition of 85 µl CTAB/NaCl the Eppendorf was vortexed briefly before incubation for 20 min at 65°C, with occasional mixing. An equal volume of chloroform : isoamyl alcohol (24 : 1, v/v) was added, the contents of the Eppendorf vortexed briefly and centrifuged for 15 min. The upper aqueous phase was transferred with a sterile wide-mouthed Pasteur pipette to a fresh Eppendorf tube and an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v/v) was added. The contents were mixed by vortexing briefly before centrifugation for 12 min. The upper aqueous phase was transferred to a new Eppendorf as before and 600 µl of isopropanol was added. The Eppendorf was mixed by gently rocking back and forth in the hand until a white DNA precipitate formed. DNA was recovered by centrifugation for 2 min followed by removal of the supernatant with a long drawn-out Pasteur pipette. The pellet was washed twice with 200 µl of 70% ethanol at room temperature and allowed to air dry for 15 - 20 min after removal of the last ethanol wash. The DNA was then allowed to dissolve in 200 µl 10 : 0.1 TE buffer, pH 7.6 overnight at 37°C. This solution containing the DNA was then stored at - 40°C until required.

2.1.7.3 Method B: Modified DNA extraction method for Gram-positive species

In order to achieve successful lysis of Gram-positive species, additional steps to the above protocol were neccessary. After the growth scraped from the agar plates had been dispersed into a homogeneous suspension, 10 µl of 10 mg/ml of lysozyme and

10 µl of 10 mg/ml of achromopeptidase were added, vortex briefly, and the Eppendorf incubated in a waterbath for 30 min at 37°C. Then 350 µl of a 2% solution of sarcosyl in HTE was added and the Eppendorf vortexed briefly, incubated at 75°C for 10 mins. After this stage the contents were cooled to 37°C before 5 µl RNase was added and the extraction procedure continued as described above.

2.1.8 DNA Assay

The purity and concentration of DNA extracted from the reference strains were measured at several wavelengths using a spectrophotometer (Pye-Unicam, SP-6, 550 UV/VIS). Two quartz microcuvettes with a total volume of 500 µl and a 1 cm pathlength were used to perform the readings and the instrument was blanked using 10 : 0.1 TE buffer, pH 7.6. A 10 µl aliqout of the solution containing bacterial DNA was mixed with 490 µl of 10 : 0.1 TE buffer and pipetted into the microcuvette. Readings of absorbance at 260 nm and 280 nm were recorded for each sample (Sambrook *et al*, 1989).

2.1.9 Protein Assay

The amount of protein contaminating DNA sample following the extraction protocols outlined above was assessed using Coomassie protein assay (Pierce Chemical Company, Illinois, USA). The spectrophotometer (Pye Unicam, Unicam SP600 Series 2) was blanked for absorbance at 595 nm using 100 µl 10 : 0.1 TE buffer, pH 7.6 and 5 ml of protein assay reagent in a 5 ml plastic disposable cuvette. A standard curve was established using 100 µl of the following concentrations of Bovine serum albumin, 1 mg/ml, 5 mg/ml, 10 mg/ml and 5 ml of assay reagent and absorbance read at 595 nm. The amount of protein contaminating a sample of extracted DNA, as outlined in the

above protocols, was measured by mixing 100 µl of sample DNA solution with 5 ml of protein assay reagent and reading absorbance versus blank at 595 nm (Bradford, 1976).

2.1.10 Agarose Gel Electrophoresis

The size of the DNA fragments extracted was determined using a minigel kit (Bethesda Research Laboratories, Maryland, USA) and agarose gel electrophoresis (Voytas, 1989). A gel of 0.8% agarose was prepared from 0.64 g agarose granules melted in 80 ml of 1 x Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA) freshly prepared from a stock solution of 50 x TAE buffer. The agarose was dissolved in 1 x TAE buffer by heating and swirling the flask occasionally until all the granules had disappeared. The melted agarose was cooled to 55°C in a water bath and ethidium bromide added to a final concentration of 0.5 µg/ml. The melted agarose was poured into the gel former complete with gel comb and left to solidify. When set, the gel comb and walls of the gel former were removed and the gel was covered to a depth of 1 mm with 1 x TAE buffer. The molecular weight marker chosen was a Hind III digest of phage Lambda DNA. Following the addition of 1 µl of 10 x loading buffer to 1 µg of bacterial DNA or DNA marker, the solution was made up to 10 µl with sterile distilled water. The 10 x loading buffer contained 0.1% bromophenol blue, 30% sucrose and 5% SDS in 10 : 1 TE buffer, pH 7.6. Bacterial DNA samples were loaded into the wells in the gel using a 20 µl micropipette with one well being reserved for the molecular weight marker. When all the DNA samples had been loaded, including the marker, the gel was run at 65 V for 2 hours or until the bromophenol blue dye had migrated three quarters along the length of the gel. The DNA was visualised directly by transilluminating the gel with 254 nm UV light (UV transilluminator, model TS-36, UVP inc, California, U.S.A.). Gels were photographed using Polaroid type 667 film and a Polariod MP4 camera equiped with a Kodak 23A Wratten filter (Voytas, 1989).

2.1.11 DNA Probe Construction

Whole chromosomal DNA extracted from the reference strains was labelled with the steroid hapten digoxigenin in one of two ways.

2.1.11.1 Method A

A random priming technique (Feinberg and Vogelstein, 1983) was used to label DNA extracted and purified as detailed above with digoxigenin. Unless otherwise stated, all reagents used in the labelling procedure were supplied by Boehringer Mannheim (U.K.). An aliquot of whole chromosomal bacterial DNA was denatured by heating in a boiling waterbath for 10 min and then cooling quickly on ice for a further 5 min to prevent re-annealing of complementary strands. The following were added to a small sterile Eppendorf tube on ice: 1 µg freshly denatured bacterial DNA, 2 µl of random hexanucleotide mixture, 2 µl of dNTP mixture containing dCTP, dATP, dGTP (1 mM each), dTTP (0.65 mM) and digoxigenin-dUTP (0.35mM). This solution was made up to 19 µl with sterile distilled water then 1 µl of Klenow fragment (DNA polymerase 1, large fragment) was added and the mixture incubated in a waterbath at 37°C. The reaction was stopped after 20 hours by the addition of 2 µl 0.2 M EDTA, pH 8.0 and the labelled DNA precipitated with 2.5 µl 4 M lithium chloride and 75 µl prechilled 70% ethanol. This mixture was then left for 80 min at - 40 °C. Labelled DNA was recovered by centrifugation for 5 min, washed with 50 µl cold 70% ethanol, centrifuged for a further 5 min before drawing off the final ethanol wash with a long drawn-out Pasteur pipette. The Eppendorf tube was placed in a 37°C incubator for 20 min or until no ethanol smell could be detected. The pellet was allowed to dissolve in 50 µl 10 : 1 TE buffer, pH 7.6 for one hour at 37°C.

2.1.11.2 Method B

The modification to the above protocol involved labelling the same quantity of template DNA but in a larger reaction volume. Approximately 1 µg of heat denatured DNA was added to a small sterile Eppendorf on ice as before. To this was added; 20 µl of random hexanucleotide mixture, 20 µl of dNTP mixture containing dCTP, dATP, dGTP (1 mM each), dTTP (0.65 mM) and digoxigenin-dUTP (0.35 mM). This solution was made up to 190 µl with sterile distilled water before the addition of 10 µl of Klenow fragment and then incubated overnight in a waterbath at 37°C. The reaction was stopped after 20 hours by the addition of 20 µl 0.2 M EDTA, pH 8.0 and the labelled DNA precipitated with 25 µl of 4 M lithium chloride and 750 µl prechilled 70% ethanol. This mixture was then left for 80 min at - 40°C. Labelled DNA was recovered as before by centrifugation for 5 min, washed with 500 µl cold 70% ethanol, centrifuged for a further 5 min before drawing off the final ethanol wash with a long drawn-out Pasteur pipette. The Eppendorf tube was placed in a 37°C incubator for 20 min or until no ethanol smell could be detected and the pellet finally allowed to dissolve in 50 µl 10 : 1 TE buffer, pH 7.6, for one hour at 37°C (Boehringer Mannheim, 1993a).

2.1.12 Colony Lift

The following method was reported by Gunaratnam *et al,* (1992). A nylon membrane 82 mm in diameter (Nytran filter, NY 13 N , 0.45 mm pore size, Schleicher and Schuell, U.K.) was placed on the surface of an agar plate containing bacterial growth and left for 2 - 5 min. It was removed and placed colony-side up on Whatman No. 1 filter paper soaked in 20% SDS for 10 min. The membrane was then placed colony-side up on fresh filter paper soaked in lysing/denaturing solution (1.5 M sodium chloride; 0.5 M sodium hydroxide) for a further 10 min. Following blotting on fresh filter paper, the membrane was again placed colony-side up on filter paper soaked in neutralising solution (1.5 M

sodium chloride; 0.5 M Tris hydrochloride, pH 7.6) for 5 min. The membrane was transferred to fresh filter paper soaked in proteinase K solution (100 mg/ml in 0.5% SDS) and incubated for 30 min at 37°C. The membrane was then immersed in chloroform : isoamyl alcohol (24 : 1, v/v) for 15 min with gentle agitation and washed in running distilled water for 10 min followed by three, 2 min washes in 95% ethanol. After air drying for 20 - 30 min, the membrane was exposed to ultra violet light 254 nm for 1 min by placing it on top of a transilluminator (model TS-36, UVP inc, California, U.S.A.) to achieve covalent bonding of the single stranded DNA to the nylon fibres (Cannon *et al,* 1984; Boeringher Mannheim, 1993b) Membranes were then stored at - 20°C until required.

2.1.12.1 Modifications to the colony lift protocol for Gram-positive species

Following lifting of colonies, the membrane was placed colony-side up on Whatman No. 1 filter paper soaked in a mixture of achromopeptidase and lysozyme, each at 100 mg/ml in HTE pH 8.0, prewarmed to 37°C for 30 min at 37°C. The colony lift protocol was then followed as described above, although the proteinase K step was increased from a 30 min soak to one hour.

2.1.13 Prehybridisation and Hybridisation

The prehybridisation, hybridisation and stringency washes were maintained as described by Gunaratnam *et al,* 1992. Prehybridisation and hybridisation stages were completed by placing the filters in hybridisation bags (Gibco Ltd. Paisley, U.K.) containing the appropriate solution and placing the bags in a waterbath (W14, Grants, U.K.) at the required temperature. Hybridisation bags were sealed using a plastic bag sealer (Salton Vacuum Bag Sealer, Model No. 1101, Pifco Ltd., U.K.).

After being marked with pencil to indicate patient number and site the membranes were washed briefly in 2 x SSC, prepared from 20 x SSC (Appendix 1). They were then prehybridised for 2 - 4 hours at 42°C in a solution containing 50% formamide, 5 x SSC, 5 x Denhardt's solution (Appendix 1), 25 mM sodium phosphate, pH 6.5 and 0.5 mg/ml freshly denatured herring sperm DNA. Twenty ml of prehybridisation solution was used per 100 cm^2 filter. The filters were then sectioned and incubated overnight at 42°C with one of the whole chromosomal DNA probes labelled with digoxigenin (at a final concentration of 100 - 250 ng/ml labelled DNA) in hybridisation solution containing 45% formamide, 5 x SSC, 1 x Denhardt's, 20 mM sodium phosphate, pH 6.5 and 0.2 mg/ml freshly denatured herring sperm DNA. A volume of 2.5 ml of hybridisation solution was allowed for every 100 cm^2 filter. After 16 hours of hybridisation the filters were subjected to two sets of stringency washes. Firstly in 2 x SSC, 0.1 % SDS (w/v) for two washes of 5 min at room temperature with gentle shaking (Rotating Table, Stuart Scientific, U.K.). Filters were then immersed in 0.1 x SSC, 0.1% SDS (w/v) at 65°C for two washes of 15 min. The membranes were then ready for detection of digoxigenin labelled hybrids with anti-digoxigenin antibody and colour substrates.

2.1.14 Detection of Digoxigenin-Labelled Probe

All volumes given in the detection protocol are calculated for 100 cm^2 of nylon membrane and were adjusted accordingly. Membranes were washed briefly in maleic acid solution (0.1 M maleic acid, 0.15M NaCl, pH 7.5; buffer 1) before being incubated for 30 min at room temperature with 100 ml of 1% blocking solution (buffer 2). This was made from a concentrate of autoclaved 10% blocking reagent in buffer 1. (Boehringer Mannheim, U.K.) Membranes were then incubated for a further 30 min with freshly diluted 150 mU/ml (1:5000) anti-digoxigenin antibody conjugated to alkaline phosphatase in 1% buffer 2. Unbound conjugate was removed by washing with 100 ml buffer 1 for two 15 min washes. The procedure from this point depended on

whether detection of bound anti-digoxigenin antibody conjugate was to be accomplished with nitro blue tetrazolium (NBT) and 5'-bromo-4-chloro-3-indolyl phoshate (BCIP) or with diazonium salts and naphthol-AS phosphate compounds (West *et al*, 1990).

2.1.14.1 NBT/BCIP detection

The membranes were equilibrated for two minutes in 20 ml of the following solution: 0.1 mM Trisma base, 0.1 mM NaCl, 50 mM $MgCl_2$, pH 9.5 (buffer 3). Detection of the presence of alkaline phosphatase was achieved by placing membranes in 10 ml of freshly prepared NBT/BCIP solution which contained; 10 ml buffer 3, pH 9.5; 45 µl NBT solution (from stock solution, Appendix 1) and 35 µl BCIP solution (from stock solution, Appendix 1). The membranes were left immersed in this solution for 3 hours in the dark at 37°C. The colour reaction was stopped by immersing the membrane in 10 : 1 TE (Tris 10 mM; EDTA 1 mM, pH 8.0) for 5 min and could be enumerated immediately prior to reprobing.

2.1.14.2 Reprobing protocol used with NBT/BCIP

After enumeration of brown colony forms on the section, the membrane was placed immersed in heated N,N-dimethylformamide (DMF) at 48°C in a sealed glass jar under a fumehood for 15 min. If neccessary, this step was repeated several times to remove most of the coloured precipitate. The membrane was then rinsed thoroughly in distilled water for 5 min before being soaked in a solution of 0.2 M sodium hydroxide for 30 min at 37°C to complete probe removal. After a brief rinse in 2 x SSC, sections of membrane were prehybridised again as described above for hybridisation with another DNA probe (Boehringer Mannheim, 1993b).

2.1.14.3 Diazonium salt and naphthol-AS-phosphate detection

The membranes were equilibrated for two minutes in 20 ml of the following solution: 0.1 mM Trisma base, 0.1 mM NaCl, 50 mM $MgCl_2$, pH 8.2 (buffer 3). Detection of the presence of alkaline phosphatase was achieved by placing membranes in 10 ml of freshly prepared red or blue colour substrate solution depending on the probe that the section of membrane had been hybridised with. Red colour substrate solution comprised 10 ml buffer 3, pH 8.2 with 0.006% fast red TR and 0.02% naphthol-AS-phosphate, whereas blue colour substrate solution comprised 10 ml buffer 3, pH 8.2 with 0.03% fast blue BB and 0.02% naphthol-AS-phosphate. All colour substrate chemicals were prepared from 10% stock solutions (Appendix 1). Colour substrate was initially left in contact with the membranes for 45 min at 37°C in the dark and replaced with fresh substrate solution and allowed to develop for a further 45 min. The colour development was stopped by washing the membranes briefly in 10 : 1 TE buffer, pH 8.0 for 5 min. Membranes were then dried at room temperature and stored. Colours did not fade, but were best seen when the membranes were rehydrated in 2 x SSC.

2.1.14.4 Alkaline phosphatase inactivation between colour detections

Alkaline phosphatase was inactivated between subsequent hybridisations by washing the membranes briefly in 10 : 1 TE buffer, pH 8.0 followed by a 10 min incubation in 0.2 M EDTA, pH 8.0 at 85°C. The membranes were finally washed twice for 5 min in buffer 1 to complete removal of EDTA. The membrane was the immediately hybridised with another DNA probe and detected with a different set of colour substrates.

2.1.15 Simultaneous Hybridisation

2.1.15.1 DNA extraction and probe construction

Whole chromosomal DNA was extracted as described in Modified DNA Extraction Method (2.1.7.2) above from the following strains, *P. intermedia* FDC 581, *A. actinomycetemcomitans* FDC Y4 and *C. ochracea* NCTC 11545. Extracted DNA was finally dissolved in 200 µl 10 : 0.1 TE buffer, pH 7.6 and stored at - 20°C. A random priming technique was used to label DNA from these three strains with one of the following labels: digoxigenin, biotin and fluorescein. Protocols for labelling DNA with each of these labels were as described above and required 1 µg bacterial DNA. All reagents were supplied by Boehringer Mannheim (U.K.). DNA was denatured by heating in a boiling waterbath for 10 min and then cooling quickly on ice for a further 5 min to prevent re-annealing. The following were added to a sterile Eppendorf tube on ice: 1 µg freshly denatured whole chromosomal bacterial DNA, 2 µl of random hexanucleotide mixture, 2 µl of dNTP mixture containing dCTP, dATP, dGTP (1 mM each), dTTP (0.65 mM) and one of the following depending on the label desired; digoxigenin-dUTP, biotin-16-dUTP, fluorescein-12-dUTP (0.35 mM each). This solution was made up to 19 µl with sterile distilled water and 1 µl of Klenow fragment added before incubation in a waterbath at 37°C. The reaction was stopped after 20 hours by the addition of 2 µl 0.2M EDTA, pH 8.0 and the labelled DNA precipitated as before with 2.5 µl of 4M lithium chloride and 75 µl prechilled 70% ethanol. This mixture was then left for 80 min at - 40 °C. Labelled DNA was recovered by centrifugation for 2 min, washed with cold 70% ethanol, dried and dissolved in 50 µl 10 : 1 TE buffer, pH 7.6.

2.1.15.2 Preparation of colony lifts

Pure cultures of the three strains were incubated for two days in pre-reduced BM CMB, as given above, in the anaerobic cabinet with 80% nitrogen, 10% hydrogen and 10% carbon dioxide at 37°C. Aliquots of 500 ml were taken from these broths and serial dilutions made in RTF. One set of dilutions comprised a mixture of equal amounts of all three broths, the remaining dilutions each contained growth from only one of the broths. Aliquots of these were then spread on TSBA plates and anaerobically incubated for 7 days at 37°C. Mixed TSBA plates at 10^{-4} dilution were selected because colony counts were estimated to be in the region of 300 to 500. Colony lifts of the mixed growth plates were prepared using nylon filters as described by Gunaratnam *et al* (1992) and single stranded DNA covalently bound to the filters following air drying by crosslinking for 3 min with 254 nm UV light from a transilluminator. Filters were then stored at - 20°C prior to hybridisation. The TSBA plates containing pure growth from the BM CMB at 10^{-4} dilution were used for colony counting to estimate the number of colony forming units of each of the three species at this dilution.

2.1.15.3 Prehybridisation and hybridisation

The colony lifts were prehybridised as described above for 2 hours at 42°C. The filters were then incubated overnight at 42°C in hybridisation solution, as described above, containing 45% formamide, 5 x SSC, 1 x Denhardt's, 20 mM sodium phosphate, pH 6.5 and 0.2 mg/ml freshly denatured herring sperm DNA, to which had been added the following freshly denatured DNA probes: *C. ochracea* DNA labelled with digoxigenin, *A. actinomycetemcomitans* DNA labelled with biotin and *P. intermedia* DNA labelled with fluorescein. Probes were at a final concentration of 50 ng/ml labelled DNA and a volume of 2.5 ml was allowed for every 100 cm^2 filter. Filters were then subjected to stringency washes, first in 2 x SSC, 0.1% SDS (w/v) for two washes of 5 min at room

stringency washes, first in 2 x SSC, 0.1% SDS (w/v) for two washes of 5 min at room temperature and then in 0.1 x SSC, 0.1% SDS (w/v) at 68°C for two washes of 15 min. As multiple detections were to be performed, the filters were subjected to UV-crosslinking for 3 min with 254 nm UV light to fix the DNA:DNA hybrids and avoid melting of the hybrids at later stages of the protocol (Boehringer Mannheim, 1993c).

2.1.15.4 Detection

The detection of the DNA hybrids was in three stages, each detecting the presence of one of the three probes at a time and resulting in a different colour being deposited at the sites of hybridisation, indicating the position of complementary target DNA at the site of the original colony. Volumes are given for each 100 cm^2 filter and colour substrate chemicals obtained from Sigma, U.K.

2.1.15.4.1 Detection of digoxigenin-labelled probe

Filters were washed briefly in maleic acid solution (0.1 M maleic acid, 0.15 M NaCl, pH 7.5. buffer 1) before being incubated for 30 min at room temperature with 100 ml of 1% blocking solution (buffer 2). This was made from a concentrate of autoclaved 10% blocking reagent in buffer 1. (Boehringer Mannheim, U.K.) Filters were then incubated for a further 30 min with freshly diluted 150 mU/ml (1:5000) anti-digoxigenin antibody conjugated to alkaline phosphatase in 1% buffer 2. Unbound conjugate was removed by washing with 100 ml buffer 1 for 2 x 15 min. The filters were equilibrated for two minutes in 20 ml of the following solution: 0.1 mM Trisma base, 0.1 mM NaCl, 50 mM $MgCl_2$, pH 8.2 (buffer 3). Detection of the presence of alkaline phosphatase was achieved by placing filters in 10 ml of freshly prepared 'green' colour substrate solution. This comprised 10 ml buffer 3, pH 8.2 with 0.03% fast blue BB and 0.02% naphthol-AS-GR-phosphate for 45 min at 37°C (Appendix 1). Colour substrate was replaced

with 10 ml of fresh substrate solution after this time and allowed to develop for a further 45 min. Alkaline phosphatase was inactivated as above (2.1.14.4).

2.1.15.4.2 Detection of biotin-labelled probes

Filters were washed briefly in buffer 1, pH 7.5 before being incubated for 30 min at room temperature with 100 ml of 5% buffer 2. Then incubated for a further 30 min with freshly diluted 150 mU/ml (1:5000) streptavidin-alkaline phosphatase conjugate in 5% buffer 2. Unbound conjugate was removed by washing with 100 ml buffer 1 for two washes of 15 min. The filters were equilibrated for two minutes in 20 ml of buffer 3. Detection of biotin-streptavidin-alkaline phosphatase complexes was achieved by placing filters in 10 ml of freshly prepared 'red' colour substrate solution. This comprised 10 ml buffer 3, pH 8.2 with 0.006% fast red TR and 0.02% naphthol-AS-phosphate for 45 min at 37°C (Appendix 1). Colour substrate was replaced with 10 ml of fresh substrate solution and allowed to develop for a further 45 min. Alkaline phosphatase was inactivated as above (2.1.14.4).

2.1.15.4.3 Detection of fluorescein-labelled probes

Filters were washed briefly in buffer 1, pH 7.5 before being incubated for 30 min at room temperature with 100ml of 1% blocking solution (buffer 2) and then incubated for a further 30 min with freshly diluted 150 mU/ml (1:5000) anti-fluorescein antibody conjugated to alkaline phosphatase in 1% buffer 2. Unbound conjugate was removed by washing with 100 ml buffer 1 for two washes of 15 min. The filters were equilibrated for two minutes in 20 ml of buffer 3. Detection of alkaline phosphatase complexes was achieved by placing filters in 10 ml of freshly prepared 'blue' colour substrate solution. This comprised 10 ml buffer 3, pH 8.2 with 0.03% fast blue BB and 0.02% naphthol-AS-phosphate for 45 min at 37°C (Appendix 1). Colour substrate was replaced with

10 ml of fresh substrate solution and allowed to develop for a further 45 min. The final colour reaction was stopped by washing the filters in 10 : 1 TE buffer, pH 8.0. Filters were then dried at room temperature and stored. As before, colours did not fade, but were best seen when the membranes were rehydrated in 2 x SSC.

2.2 CLINICAL PROCEDURES

2.2.1 Patients

Patients who were seropositive for the Human Immunodeficiency Virus (HIV) participating in the study were referred to the Edinburgh Dental Hospital from a variety of sources. Patients were referred from the Department of Genito-Urinary Medicine at the Royal Infirmary of Edinburgh and the Infectious Diseases Unit at the City Hospital of Edinburgh, either for specific treatment of oral conditions due to their immunodeficiency or for regular observation should any oral conditions associated with HIV infection arise. Other patients were self-referred, seeking general dental treatment as a result of difficulties in finding a general dental practitioner. The nature of periodontal disease in HIV seropositive subjects and the aims of this study were explained to the patients, after which they were invited to participate.

Control patients, assumed to be HIV seronegative, responded to a poster displayed in the Edinburgh Dental Hospital requesting volunteers to take part in a study of periodontal disease. The Edinburgh Dental Hospital is primarily a teaching hospital although it does provide a specialist consultant service for referrals from general dental practitioners. It also serves as an acute care centre for the local population that may not have a general dental practitioner of their own.

2.2.2 Initial Visit

Each patient was randomly assigned to one examiner at baseline and continued to see that clinician on all subsequent visits. A medical and social history was taken for all patients at the initial visit (Appendix 2). Following a thorough oral examination, dichotomous indicators of plaque, redness, necrosis, sequestration and suppuration were recorded at six sites for all teeth present with the exception of third molars (Haffajee *et al*, 1984). Supragingival plaque was removed from the mesiobuccal surfaces of all teeth by means of Gracey curettes and a subgingival plaque sample taken from the mesiobuccal site of all teeth with the exception of third molars. Each plaque sample was deposited in 5 ml of RTF contained in a plastic disposable bijou marked with patient number and site. Once all the plaque samples were collected the bijoux were taken to the laboratory for dispersal, dilution and plating as outlined below. Pocket depth measurements and position of the amelocemental junction were recorded with a Williams probe at six sites for all teeth with the exception of third molars.

2.2.3 Laboratory Procedure

Due to the nature of the samples it was decided not to use a sonicating tip to achieve dispersal of the plaque sample, but instead immerse the sealed bijoux containing the plaque samples in a ultrasonic waterbath. This would remove the health risk posed by the formation of a potentially harmful aerosol if a sonicating tip and open containers were used.

The bijou containing the plaque sample was placed in an ultrasonic water bath, until the level of the RTF inside the bijou was beneath the level of water in the bath, and sonicated for 60s. After mixing by inverting the bijou several times, the dispersed plaque sample was serially diluted in 10-fold dilutions of RTF. Using a 1 ml

micropipette (Gilson Ltd., U.K.), 500 µl of the dispersed plaque sample was transferred under a continuous flow of oxygen-free nitrogen to a fresh bijou containing 4.5 ml RTF. The top of this bijou was secured and it was inverted several times before 500 µl of this dilution was removed and transferred under oxygen-free nitrogen to another fresh bijou containing 4.5 ml RTF and again the contents mixed thoroughly by inverting. Plaque samples taken from the mesiobuccal sites of molar teeth were diluted one stage further to allow for larger samples from these teeth. Aliquots of 100 µl of the neat, 10^{-1}, 10^{-2} and 10^{-3} dilutions of plaque samples, were spread on TSBA plates marked for patient number and site. These plates were then incubated in the anaerobic cabinet at 37°C. When confluent growth was established on the agar surface after a period of between 5 to 7 days, the growth from agar plates with a total colony count of between 100 and 2000 was lifted on to nylon membranes using method A described in Materials and Methods above. The colony lifts were then stored at -20°C until required. When a number of patient samples had been collected and colony lifted, hybridisations were completed as described above, at first using NBT/BCIP substrates to detect alkaline phosphatase and subsequently naphthol-AS phosphates and diazonium salts.

2.2.4 Recall and Subsequent Visits

HIV seropositive patients were instructed to return immediately if they suffered any gingival discomfort, pain, bleeding or had any concerns regarding their periodontal or oral health between recall visits. Recalls were arranged initially only for HIV seropositive patients on a three monthly basis. At these visits, clinical measurements and dichotomous indicators of periodontal disease were recorded for the same six sites of teeth present with the exception of third molars as before. A subgingival plaque sample was collected only from those sites calculated to have lost 3 mm or more of attachment between visits (see Calibration study, 2.2.5). These sites were deemed to have been active in the intervening period. A subgingival plaque sample was also taken from any

site that had the same final attachment level but that had suffered no additional loss between visits, this site was designated as a control site. Recall visits were later organised on 9 to 18 month basis for HIV seronegative patients and all measurements were repeated as detailed above for HIV seropositive patient recalls. Two clinicians were responsible for collection of all clinical measurements and plaque samples from both the HIV seropositive and HIV seronegative patients.

2.2.5. Calibration Study

2.2.5.1 Aim

This study was undertaken to calibrate both clinical examiners and give estimates of false positive rates when using a Williams probe to determine attachment level loss.

2.2.5.2 Design

The calibration study was conducted in two parts and comprised cross-sectional and longitudinal measurements. The cross-sectional measurements were intended to give an estimate of intra- and inter-examiner correlation and were conducted on two patients. Both examiners measured pocket depths and levels of ACJ, of the maxillary teeth only, in the same two patients on two separate occasions. The time between paired measurements by one examiner was one hour and, as no real change in attachment level could occur within this period, analysis of the data would also reveal a false positive rate. The longitudinal measurements were analysed retrospectively on a total of three patients who had attended each examiner on at least three occasions within a six month period. Statistical analysis of these data would provide an estimate of intra-examiner correlation.

2.2.5.3 Results

2.2.5.3.1 Cross-sectional data

Cross-sectional data is given in Table 3. The examiners in this study were in absolute agreement in 70.5% of sites measured in the cross-sectional measurements. Inter-examiner agreement of attachment level was 98-100% at ∓ 2 mm.

2.2.5.3.2 Longitudinal data

The longitudinal data is given in Table 4. Total number of sites reviewed over three visits was 498 and the total number of comparisons was 1498. From the results the intra-examiner agreement was 90% ∓ 1 mm and 98% ∓ 2 mm. The number of sites found in these three patients to have lost 3 mm attachment was six therefore the rate of activity found in these patients from 1498 measurements was 0.6%. Results reported by Best *et al* (1990) are included for comparison.

Table 3 Calibration Study Cross-sectional Data

	Mean %	Best *et al*, 1990 Mean %
Absolute agreement	70.5	44.3
Difference of 1mm	27.7	40.9
Difference of 2mm	1.8	11.5
Difference of 3mm	-	2.3
Difference of 4mm	-	1.0
Number of sites	168	4963

Table 4 Calibration Study Longitudinal Data

	Range %	Mean %	Best *et al*, 1990 Mean %
Absolute agreement	38 - 63	48	41.6
Difference of 1mm	34 - 47	42	44.0
Difference of 2mm	3 - 11	8	11.7
Difference of 3mm	0 - 3	1.3	2.2
Difference of 4mm	0 - 1	0.7	0.5
Number of sites		498	3604

2.3 STATISTICAL ANALYSES

All statistical analyses were achieved by using a SPSS statistical package available on Edinburgh University mainframe computer. In the majority of cases the data of parameters studied were not normally distributed and transformations proved unsuccessful, therefore statistical analysis of data was achieved using various non-parametric tests.

CHAPTER 3

RESULTS

3.1 LABORATORY RESULTS

3.1.1 DNA Extraction and Spectrophotometer Assay

Table 5 gives details of bacterial DNA extracted from reference strains using method B, with modifications for the Gram-positive species used for *S. sanguis*. Spectrophotometer readings of absorbance at 260 nm and 280 nm are also included, as are the ratios of 260 : 280. These extractions were chosen to construct DNA probes because of good 260 : 280 ratios, indicating highly purified preparations of DNA.

Table 5 DNA Assay

	Absorbance			
	260 nm	280 nm	ratio	yield (µg)
A. actinomycetemcomitans	0.134	0.077	1.74	67.0
P. gingivalis	0.347	0.205	1.69	173.5
P. intermedia	0.042	0.025	1.68	21.0
C. rectus	0.240	0.144	1.67	120.0
B. forsythus	0.069	0.042	1.64	34.5
C. ochracea	0.094	0.057	1.65	47.0
V. parvula	0.037	0.021	1.76	18.5
F. nucleatum	0.193	0.11	1.75	96.5
S. sanguis	0.096	0.057	1.68	48.0

3.1.2 Protein Assay

Using the Pierce Coomassie protein assay, negligible quantities of protein were found in bacterial DNA extracted using method B.

3.1.3 Agarose Gel Electrophoresis

The size of bacterial DNA fragments extracted was estimated using AGE. The bulk of DNA extracted was found to be in the region of 23.1 kb or larger. This is in agreement with previous results reported by Smith *et al* (1989b). Figure 3 shows results from an AGE to estimated the size of DNA fragments after shearing. Extracted DNA was sheared by passing through a 25 gauge needle four times. Lane 1 contains a Hind III digest of phage λ-DNA with eight bands that represent DNA fragment sizes of 23.13 kb, 9.42 kb, 6.56 kb, 4.36 kb, 2.32 kb, 2.03 kb, 0.56 kb and 0.13 kb respectively. Lanes 2 through 9 contain DNA extracted from the following *A. actinomycetemcomitans, C. ochracea, B. forsythus, C. rectus, V. parvula, P. gingivalis, F. nucleatum* and *P. intermedia.* The results of this experiment indicate that the size of the DNA from these organisms after shearing is generally 2.32 kb or smaller. Figure 4 is a photograph of a gel containing digoxigenin-labelled *C. ochracea* DNA and the phage λ-DNA marker as before. This shows that the size of the digoxigenin-labelled DNA is comparable to unlabelled DNA after shearing, being 2 kb or less. The absence of RNA fluorescence at or beyond the gel front is a further indication of the purity of the extracted DNA.

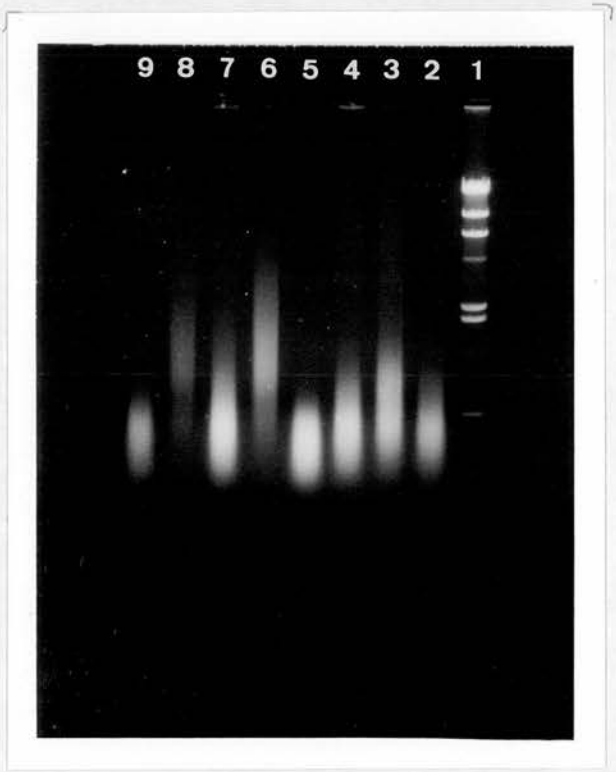


Figure 3 AGE of Sheared Bacterial DNA

Lane 1 Hind III digest of phage Lambda DNA,

Lane 2 *A. actinomycetemcomitans*, Lane 3 *C. ochracea*,

Lane 4 *B. forsythus*, Lane 5 *C. rectus*, Lane 6 *V. parvula*,

Lane 7 *P. gingivalis*, Lane 8 *F. nucleatum*, Lane 9 *P. intermedia.*

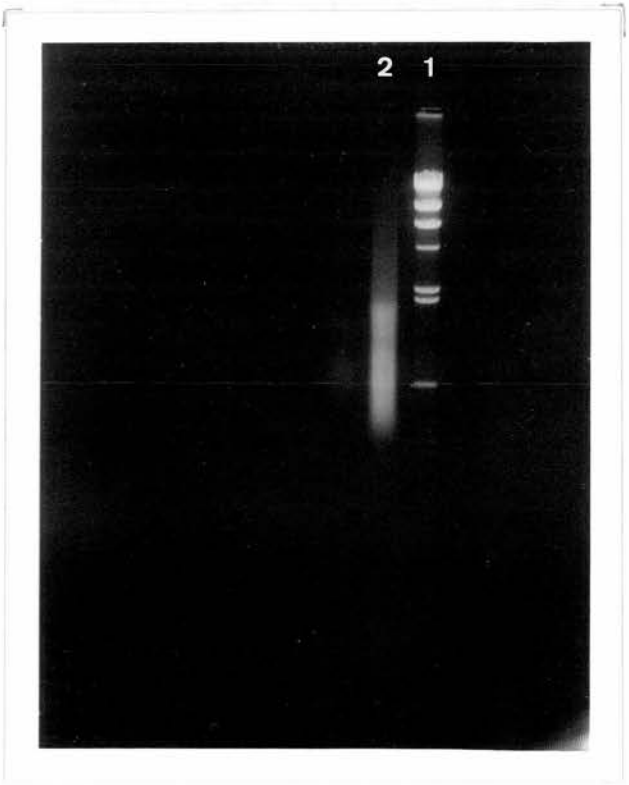


Figure 4 AGE of Digoxigenin-labelled DNA

Lane 1 Hind III digest of phage Lambda DNA,

Lane 2 digoxigenin-labelled *C. ochracea* DNA

3.1.4 DNA probes

Figure 5 shows ten-fold dilutions of labelled DNA in concentrations from 100 ng to 10 pg detected with a range of colours possible by altering the combination of napthol-AS-phosphates and diazonium salts. The colours produced are from the left Fast Red, Green, Fast Blue, and Fast Brown. This experiment also compared the colours produced by reducing the pH of the colour substrate buffer from 9.5 to 8.2. Improved definition and clarity can be achieved by lowering the pH from 9.5 (right hand strip of each pair) to 8.2 (left hand strip). Figure 6 shows the result of a labelling reaction which was detected with napthol-AS-phosphate and fast red TR diazonium salt.

Figure 5 Range of Colours using Colour Substrates

1. Fast Red TR and Naphthol-AS-phosphate
2. Fast Blue BB and Naphthol-AS-GR-phosphate
3. Fast Blue BB and Naphthol-AS-phosphate
4. Fast Brown and Naphthol-AS-phosphate

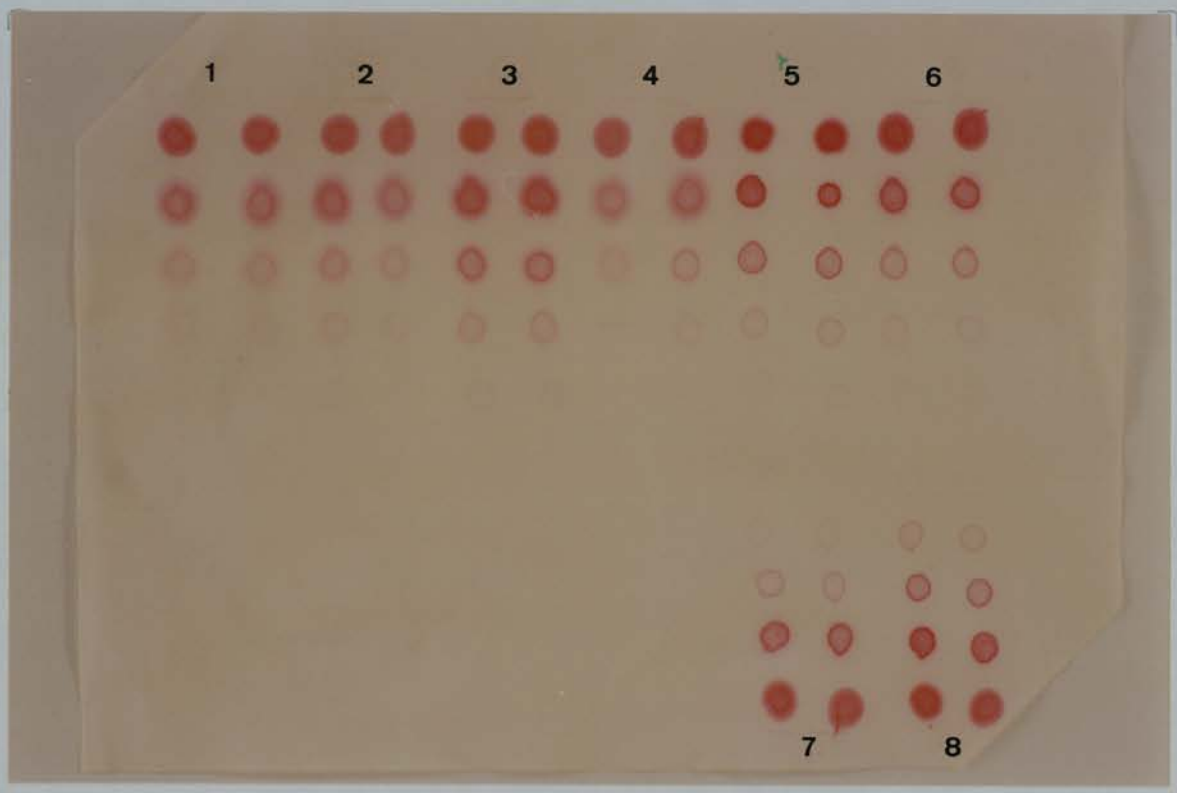


Figure 6 Labelling Reaction Detected with Fast Red TR and Naphthol-AS-phosphate

1. *A. actinomycetemcomitans*, 2. *B. forsythus*, 3. *C. ochracea*,
4. *C. rectus*, 5. *P. intermedia*, 6. *P. gingivalis*, 7. *V. parvula*,
8. *F. nucleatum*.

3.1.5 Colony Lifts

Control colony lifts were included with every hybridisation reaction and detected with either NBT/BCIP or red and blue colour substrates. Figures 7 and 8 show a selection of colony lifts detected.

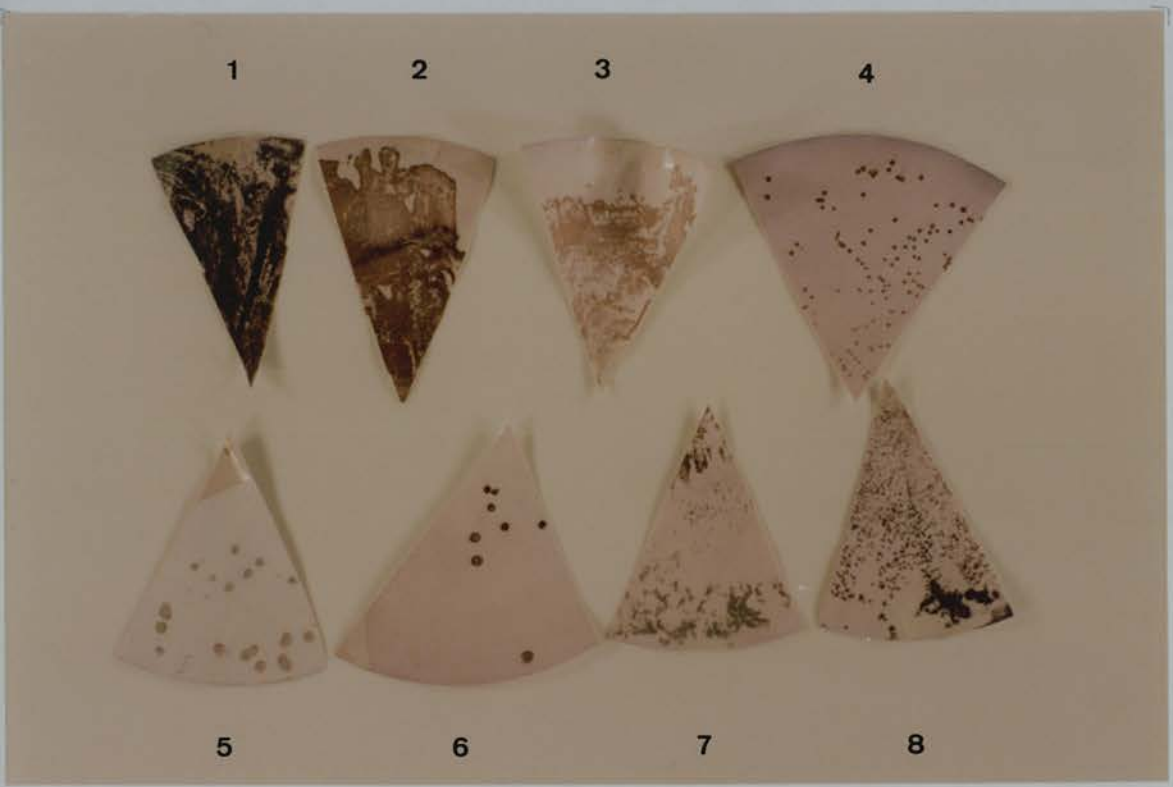


Figure 7 Colony Lifts Detected with NBT/BCIP

1. *A. actinomycetemcomitans*, 2. *C. ochracea*, 3. *P. gingivalis*,
4. *V. parvula*, 5. *P. intermedia*, 6. *C. rectus*, 7. *F. nucleatum*
8. *B. forsythus*.

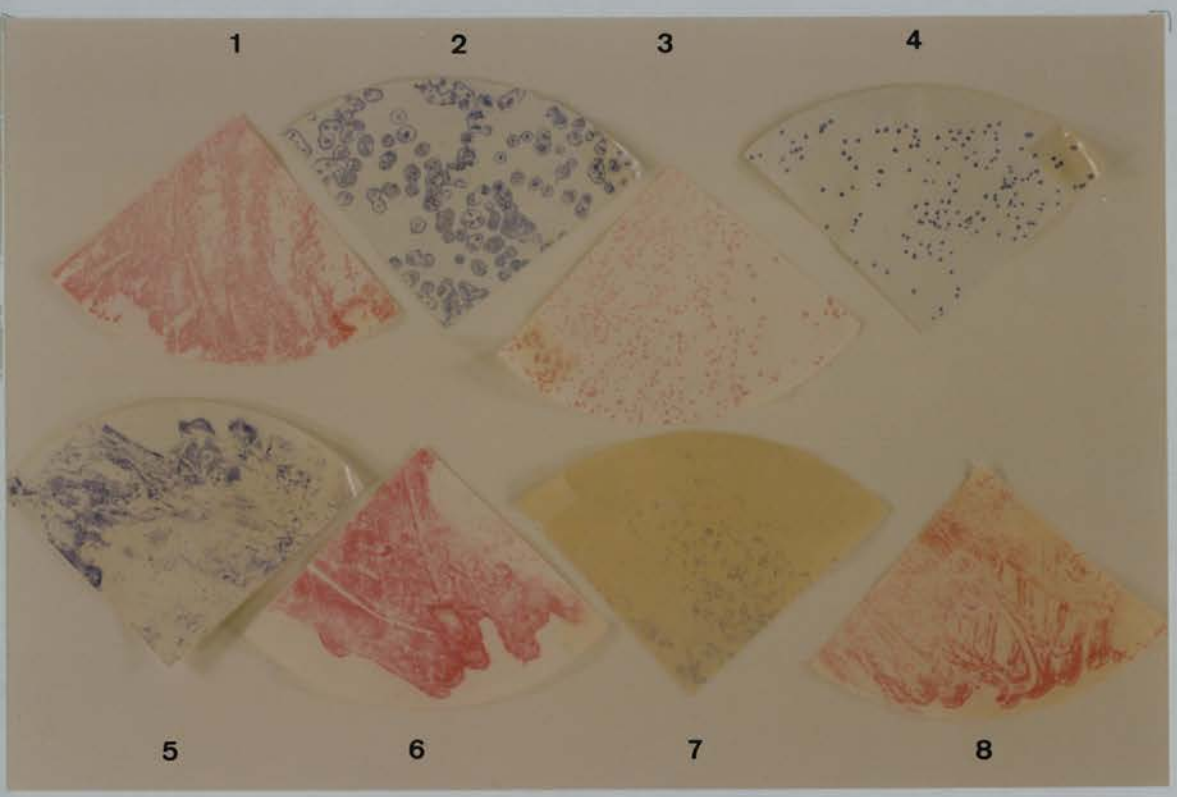


Figure 8 Colony Lifts Detected with Colour Substrates

1. *A. actinomycetemcomitans*, 2. *P. gingivalis*, 3. *B. forsythus*,
4. *V. parvula*, 5. *P. intermedia*, 6. *C. ochracea*, 7. *F. nucleatum*
8. *C. rectus* .

3.1.5.1 Selection of periodontal species to be enumerated

Only species that had been validated by Gunaratnam *et al* (1992) were considered for use in this study and every attempt was made to purchase equivalent strains to those validated by these authors. Due to restrictions of time and manpower, it was originally decided to limit the number of species investigated to those nine detailed in Materials and Methods. These species were selected because they featured prominently in the literature as currently suspected periodontal pathogens or beneficial species. However during test hybridisations it became apparent that *S. sanguis* colonies were not lysed consistently using the colony lift protocol described by Gunaratnam *et al* (1992). This was attributed to the resilience of the Gram-positive cell wall possessed by this organism. Although lysis of Gram-positive bacteria could be improved by additional steps to the colony lift, this resulted in elution from the surface of the filter of Gram-negative species on a mixed growth colony lift. It was therefore decided to concentrate only on the Gram-negative species selected. Test hybridisations with *F. nucleatum* gave inconsistent results, although the colony lift method was not believed to be at fault in this case. Colony lifts of patient samples were hybridised with the *F. nucleatum* probe, however detection of *F. nucleatum* measured by the strength of signal on the positive control colony lifts during these hybridisations remained inconsistent and at best very weak. For these reasons the results reported do not include *S. sanguis* or *F. nucleatum* and are for the remaining seven Gram-negative species only.

3.1.6 Stripping Procedure

Figure 9 shows a colony lift that demonstrates some of the problems caused by reprobing when alkaline phosphatase is detected with NBT/BCIP. This colony lift was prepared from an agar plate that contained a mixture of *A. actinomycetemcomitans* and *C. ochracea* growth and was first hybridised with a digoxigenin-labelled

A. actinomycetemcomitans DNA probe and detected with NBT/BCIP. After enumeration of colony forms the brown precipitate and probe were stripped using the protocol outlined in Materials and Methods. The lift was then hybridised with a digoxigenin-labelled *C. ochracea* DNA probe and detected with NBT/BCIP. Whereas the brown precipitate produced at sites of hybridisation with the *C. ochracea* probe is clearly visible, blue ghost colony forms from the first detection remain visible after stripping. It is also possible to make out the effect that light can have on a colony lift detected with NBT/BCIP. An area exposed to light during the detection has lightened, leaving dark outlines of two separate lifts overlying the one photographed.

Figure 9 Removal of NBT/BCIP Precipitate with DMF

3.1.7 Patient Samples

Detection of the probe species on colony lifts prepared from primary isolation plates using colour substrates is illustrated in Figure 10. These two quadrants of filter were first hybridised with *C. rectus* digoxigenin-labelled DNA probe and detected with 'fast red' colour solution then hybridised with *P. intermedia* digoxigenin-labelled DNA probe and detected with 'fast blue'. The remaining quadrants from these sites were hybridised with one of the following pairs of digoxigenin-labelled DNA probes
A. actinomycetemcomitans and *P. gingivalis*, *C. ochracea* and *V. parvula*,
F. nucleatum and *B. forsythus*.

Figure 10 Patient Samples Detected with Colour Substrates

Red colony forms represent areas where the *C. rectus* DNA probe has hybridised and blue colony forms represent areas where the *P. intermedia* DNA probe has hybridised.

3.1.8 Simultaneous Hybridisations

After anaerobic incubation for seven days, TSBA plates with growth from aliquots of the 10^{-4} dilution of pure and mixed dilutions of CMB, were selected for colony counting and colony lift respectively. Triplicate TSBA plates containing growth of separate species at the 10^{-4} dilution of the CMB were counted using a colony counter. From these results mean total colony counts and standard error for each species at this dilution were calculated (Table 6). The mean total colony count represents the mean number of CFU's for each species contained in a 100 µl aliquot of a 10^{-4} dilution of each CMB. The estimated total colony count was calculated by adding these three means to give an estimate of the total colony count of all three species on a mixed growth TSBA plate (Table 6). Mixed TSBA plates at 10^{-4} dilution, were colony lifted, simultaneously hybridised and colour detected as described in Materials and Methods. Coloured colony forms on the lifts represented areas where the DNA probes to these species had successfully hybridised with single-stranded target DNA fixed to the nylon colony lift (Figure 11 a,b). Total colony counts of mixed growth TSBA plates with green, blue and red colony forms, representing *C. ochracea, P. intermedia* and *A. actinomycetemcomitans* colonies respectively, are given in Table 7. The mean total colony count of all coloured colony forms on each lift, together with mean total colony count and standard error for each species are also given.

Table 6 Colony Counts for Pure Growth TSBA Plates

	plate			mean	SE
	1	2	3		
C. ochracea	146	132	125	134.3	6.18
P. intermedia	40	61	48	49.7	6.12
A. actinomycetemcomitans	24	31	34	29.7	2.94
Estimated total colony count				213.4	

Table 7 Colony Counts for Mixed Growth TSBA Plates Enumerated by Simultaneous Hybridisation and Multicolour Detection

	plate						mean	SE
	1	2	3	4	5	6		
C. ochracea	152	127	114	175	163	131	143.7	9.56
P. intermedia	36	39	39	43	59	36	42.0	3.56
A. actinomycetemcomitans	29	19	16	31	32	30	26.2	2.80
Mean total colony count	217	185	169	249	254	197	211.8	14.10

Figure 11a Simultaneous Hybridisation and Multicolour Detection

Green colony forms represent *C. ochracea* DNA

Red colony forms represent *A. actinomycetemcomitans* DNA

Blue colony forms represent *P. intermedia* DNA

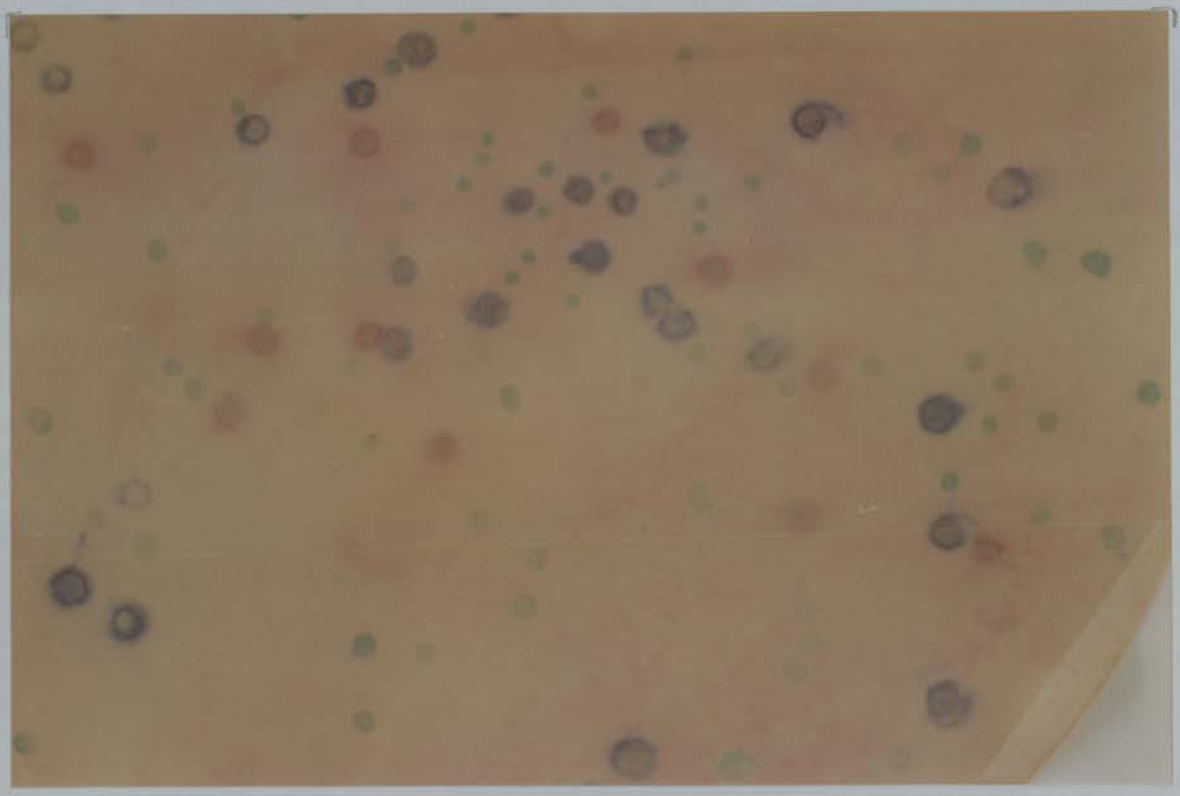


Figure 11b Simultaneous Hybridisation and Multicolour Detection

(Higher power view of colony lift in Figure 11a)

Green colony forms represent *C. ochracea* DNA

Red colony forms represent *A. actinomycetemcomitans* DNA

Blue colony forms represent *P. intermedia* DNA

3.2 PATIENT POPULATION

A total of 29 HIV seropositive patients were recruited from the Department of Genito-Urinary Medicine at the Royal Infirmary of Edinburgh and the Infectious Diseases Unit at the City Hospital of Edinburgh to take part in this study. All patients were diagnosed HIV antibody positive by ELISA and confirmed by Western Blot. This group comprised 23 males and 6 females, with a mean age of 33.2 years, s.d 8.47 (Table 8). Twenty patients had acquired HIV by homosexual or bisexual contact, five by heterosexual contact and four patients during IVDA. The median number of years since diagnosis of HIV infection at baseline examination was three, although this ranged from a few months to eight years. Twelve patients had a CDC classification of stage II and seventeen stage IV (Table 9). Many of these patients were on a wide range of medication and presented with a number of HIV-related and unrelated illnesses. Table 10 gives the occurrence of systemic and oral conditions believed to be related to immunosupression caused by HIV infection. Five HIV seropositive subjects gave a history of Herpes zoster which was the most common systemic manifestation of HIV infection in this sample representing 17.4% of the population studied. Four patients had a history of Herpes simplex, three *Pneumocystis carinii* pneumonia and two subjects had a history of Kaposi's sarcoma. Other systemic conditions recorded at first visit for these patients included appendicitis, peptic ulcer and diabetes insipidus. The most common oral manifestation of HIV infection was oral hairy leukoplakia which occurred in eleven (37.9%) of HIV seropositive subjects. Of the seven cases of candidiasis reported five were pseudomembraneous candidiasis, one was atrophic candidiasis and one patient had both pseudomembraneous and atrophic candidiasis at the same time. Other oral signs believed to be related to HIV infection included four cases of recurrent oral ulceration, two with Herpes simplex and one oral Kaposi's sarcoma. Table 11 gives the percentage of HIV seropositive subjects on antimicrobial, antiviral and antifungal medication at first visit. Fifteen patients were recieving azidothymidine, three acyclovir

and one patient was taking dideoxyinosine. A total of sixteen patients were receiving antimicrobial therapy at the first visit, eleven subjects were taking pentamidine either prophylactically or for the treatment of *Pneumocystis carinii* pneumonia. Twelve patients were on a single course of antimicrobials, eight on pentamidine only, and four patients were taking a single course of either penicillin V, metronidazole, trimethoprim or doxycycline. Four patients were on a combination of antimicrobials at first visit, two patients were receiving pentamidine and metronidazole, one patient pentamidine and penicillin V, and one patient was taking metronidazole and erythromycin. Of those patients on antifungals, four were taking fluconazole and one amphoteracin. Other medications recorded included three subjects on methadone with Valium, temazepam, codeine, aspirin, Tagamet, dapsone and Lotomil being taken by one patient each.

Twenty-seven patients volunteered to act as a control group and comprised 18 males and 9 females. Two male subjects were homosexuals, but had recently tested negative for HIV. The remaining 25 patients were assumed to be HIV negative. The mean age of this group was 35.5 years (s.d 11.06) (Table 8).

Table 8 Patient Statistics 1

	HIV +			HIV -		
	n	AGE	sd	n	AGE	sd
Male	23	35.2	8.37	18	34.4	9.12
Female	6	25.7	2.80	9	37.7	14.59
Total	29	33.2	8.47	27	35.5	11.06

Table 9 Patient Statistics 2

	HIV +			HIV +		HIV -	
	n	%		n	%	n	%
homo/bi	20	69.0	CDC II	12	41.4		
hetero	5	17.3	CDC IV	17	58.6		
IVDA	4	13.7	smoking	14	48.3	15	55.6
			non smoking	15	51.7	12	44.4

homo/bi	homosexual/bisexual	CDC II	CDC stage II
hetero	heterosexual	CDC IV	CDC stage IV
IVDA	intravenous drug abuser		

Table 10 Medical History of HIV Seropositive Subjects at First Visit

		n	%
Systemic	Herpes zoster	5	17.4
	Herpes simplex	4	13.8
	Pneumocystis	3	10.3
	Kaposi sarcoma	2	6.9
Oral	Oral Hairy Leukoplakia	11	37.9
	Candida	7	23.3
	Recurrent Oral Ulcers	4	13.8
	Herpes simplex	2	6.9
	Kaposi sarcoma	1	3.4

Table 11 Medication taken by HIV Seropositive Subjects at First Visit

	n	%		n	%
Antimicrobials	16	55.2	Antivirals	17	58.6
pentamidine	11	37.9	azidothymidine	15	51.7
metronidazole	4	13.8	acyclovir	3	10.3
penicillin V	2	6.9	dideoxyinosine	1	3.4
erythromycin	1	3.4			
trimethoprim	1	3.4	Antifungals	5	17.2
doxycycline	1	3.4			
			fluconazole	4	13.8
			amphoteracin	1	3.4

3.2.1 Baseline and Subsequent Visits

At baseline examination both groups had a similar mean number of teeth, i.e. 25.9 (s.d. 2.3) in the HIV seropositive group, 25.1 (s.d. 3.1) in the HIV seronegative group. Clinical data were collected on 4500 sites from the HIV seropositive patients and 4068 sites from the HIV seronegative patients at baseline examination. Subgingival plaque samples were taken from a total of 750 sites from HIV seropositive subjects and 678 sites from HIV seronegative subjects at this visit. Tables 12 and 13 give the number of HIV seropositive patients that attended recall appointments. Eighteen patients were seen on a second visit, 16 males and two females, comprising 14 homosexuals or bisexuals, two heterosexuals and two IVDA. Details of HIV seropositive patients attending third, forth, fifth and sixth visits are also given in Table 13. The mean interval between appointments was 3.6 months (s.d 0.80).

Table 12 Subsequent Visits of HIV Seropositive Subjects

	number of visits					
	1	2	3	4	5	6
HIV +						
patients	29	18	9	5	3	1
sites	4500	2814	1404	792	498	168
HIV -						
patients	27	4				
sites	4068	672	-	-	-	-

Table 13 Details of HIV Seropositive Subjects Re-attending

	visit									
	2		3		4		5		6	
	n	%	n	%	n	%	n	%	n	%
male	16	89	8	89	5	100	3	100	1	100
female	2	11	1	11	0	0	0	0	0	0
homo/bi	14	78	7	78	5	100	3	100	1	100
hetero	2	11	1	11	0	0	0	0	0	0
IVDA	2	11	1	11	0	0	0	0	0	0
CDC II	8	44	4	44	1	20	1	33	0	0
CDC IV	10	55	5	55	4	80	2	66	1	100
smoking	8	44	4	44	1	20	1	33	0	0
non smoking	10	55	5	55	4	80	2	66	1	100

3.3 STATISTICAL ANALYSES

3.3.1 Attachment Loss and Clinical Indicators

Individual histograms of mean attachment loss for each patient presented highly positively-skewed data. Log_{10} , log_{10} (x + 1) and square root transformations failed to correct for skewness and a normal distribution could not be obtained. However, if a log_{10} (x + 1) transformation of the mean attachment loss of the worst 10% of sites is plotted, abbreviated to CTENP (Imrey, 1986), a normal distribution is attainable for the HIV seronegative patients (Figure 12). This same transformation failed using data from the HIV seropositive group (Figure 13). It was therefore decided that untransformed mean attachment loss for each patient would serve as a summary statistic of attachment loss for these two patient groups. Comparisons of attachment loss between groups was achieved using mean attachment loss and a Mann-Whitney U test. Clinical indicators of periodontal disease were also not normally distributed for either groups and therefore appropriate non-parametric tests were chosen for analyses.

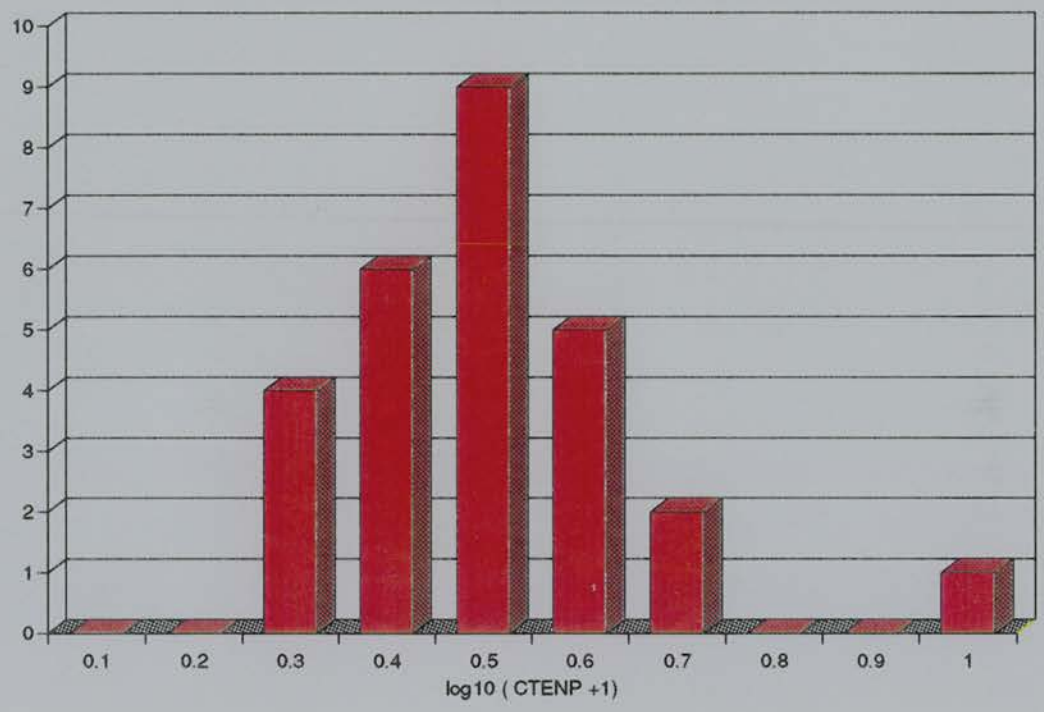


Figure 12 Frequency Distribution for $Log_{10}(x + 1)$ Transformation of CTENP for HIV Seronegative Subjects

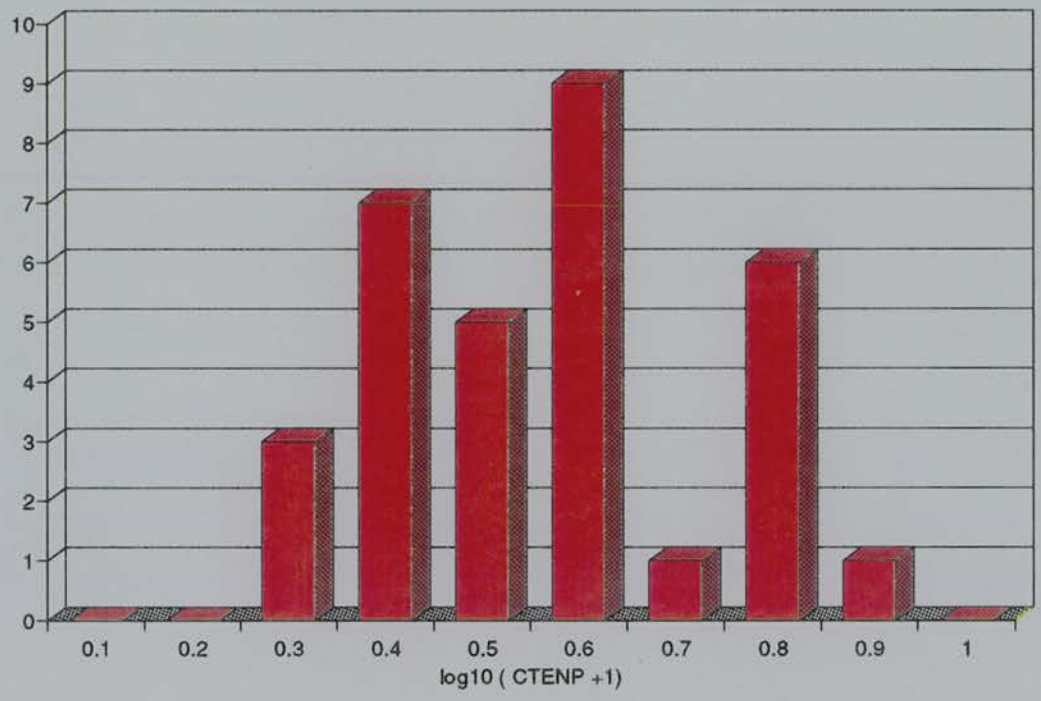


Figure 13 Frequency Distribution for $Log_{10}(x + 1)$ Transformation of CTENP for HIV Seropositive Subjects

3.3.2 Comparisons of Microbiological Data Between Patient Groups

Both prevalence and mean percentage of each of the species enumerated varied widely between patients of both groups. The mean percentage of any one of the species was not normally distributed in either the HIV seropositive or HIV seronegative group and transformation of the raw data also proved unsuccessful. Comparisons between HIV seropositive and seronegative groups were achieved using a Mann-Whitney U test. The median is included in the Tables of microbiological data as a summary statistic for the groups, and the interquartile range is given as an indication of the range of the mean percentages for the species enumerated.

3.3.3 Periodontal Parameters as Possible Predictors of HIV Disease Progression

Baseline clinical and microbiological data collected from both HIV seropositive and HIV seronegative subjects were analysed using a Kruskal Wallis test, non-parametric one way analysis of variance, to find out if HIV disease progression could be related to any of the parameters studied. Other tests used in statistical analyses are described with the data in the Results section.

3.4 CLINICAL DATA

3.4.1 Baseline Visit

3.4.1.1 Attachment loss

Figures 14 and 15 plot the mean attachment loss at baseline examination against age for HIV seronegative and HIV seropositive patients. Mean attachment loss for HIV

seronegative patients increased with age, however two HIV seronegative patients had a greater mean attachment loss for their corresponding age compared to the rest of the HIV seronegative patients in this sample (Figure 14). The relationship between mean attachment loss and age for HIV seropositive patients was considerably more complex (Figure 15), whereas some HIV seropositive patients had a similar distribution of mean attachment loss for age compared to the HIV seronegative patients in this sample, as many as fourteen HIV seropositive patients had an increased mean attachment loss for their age (Figure 16). When these two groups were compared using a Mann-Whitney U test, the HIV seropositive patients tended to have a greater mean attachment loss than HIV seronegative patients ($p = 0.099$, Table 14).

Table 14 Comparison of Mean Attachment Loss At Baseline Visit of HIV Seropositive and HIV Seronegative Subjects
(Mann-Whitney U Test).

	Median	Interquartile Range	p-value
HIV +	1.40	0.68 - 2.48	
HIV -	0.90	0.77 - 1.38	0.099

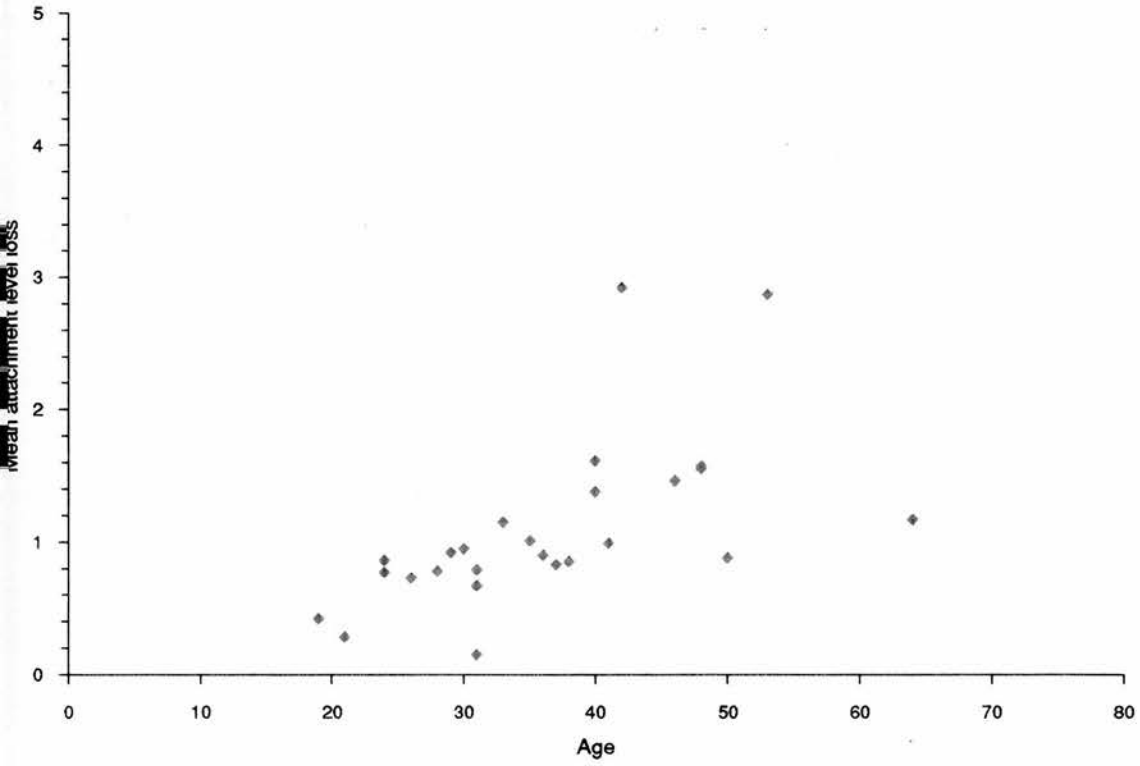


Figure 14 Mean Attachment Loss in HIV Seronegative Subjects

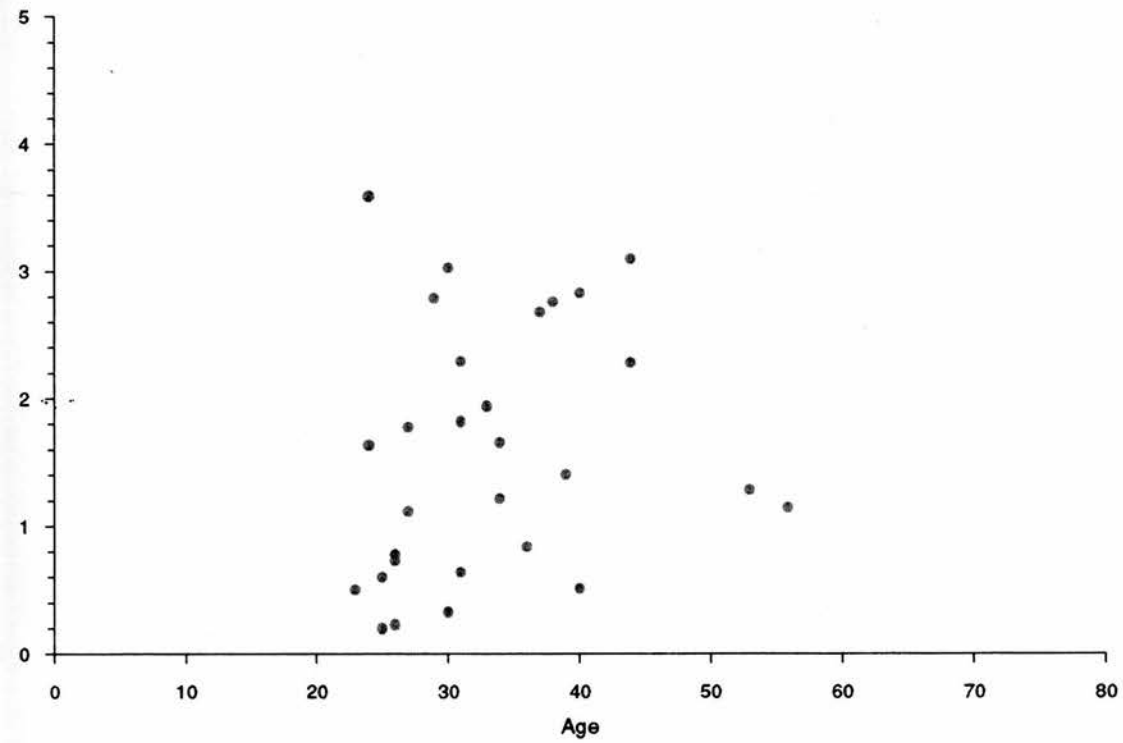


Figure 15 Mean Attachment Loss in HIV Seropositive Subjects

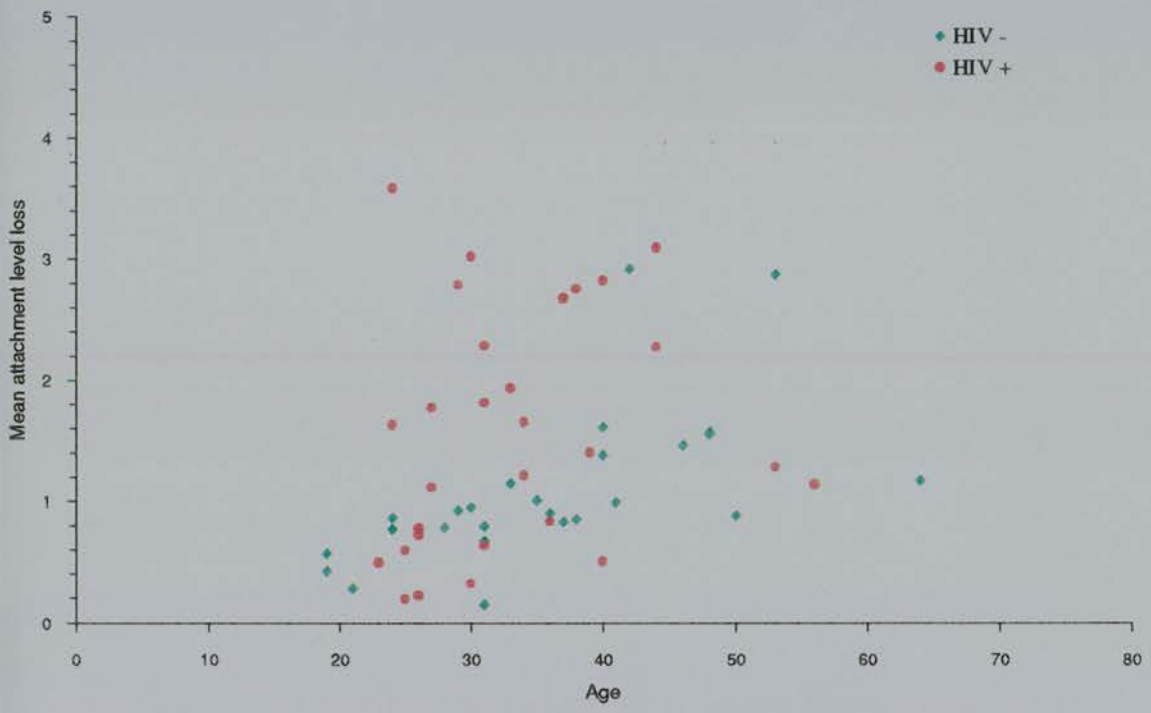
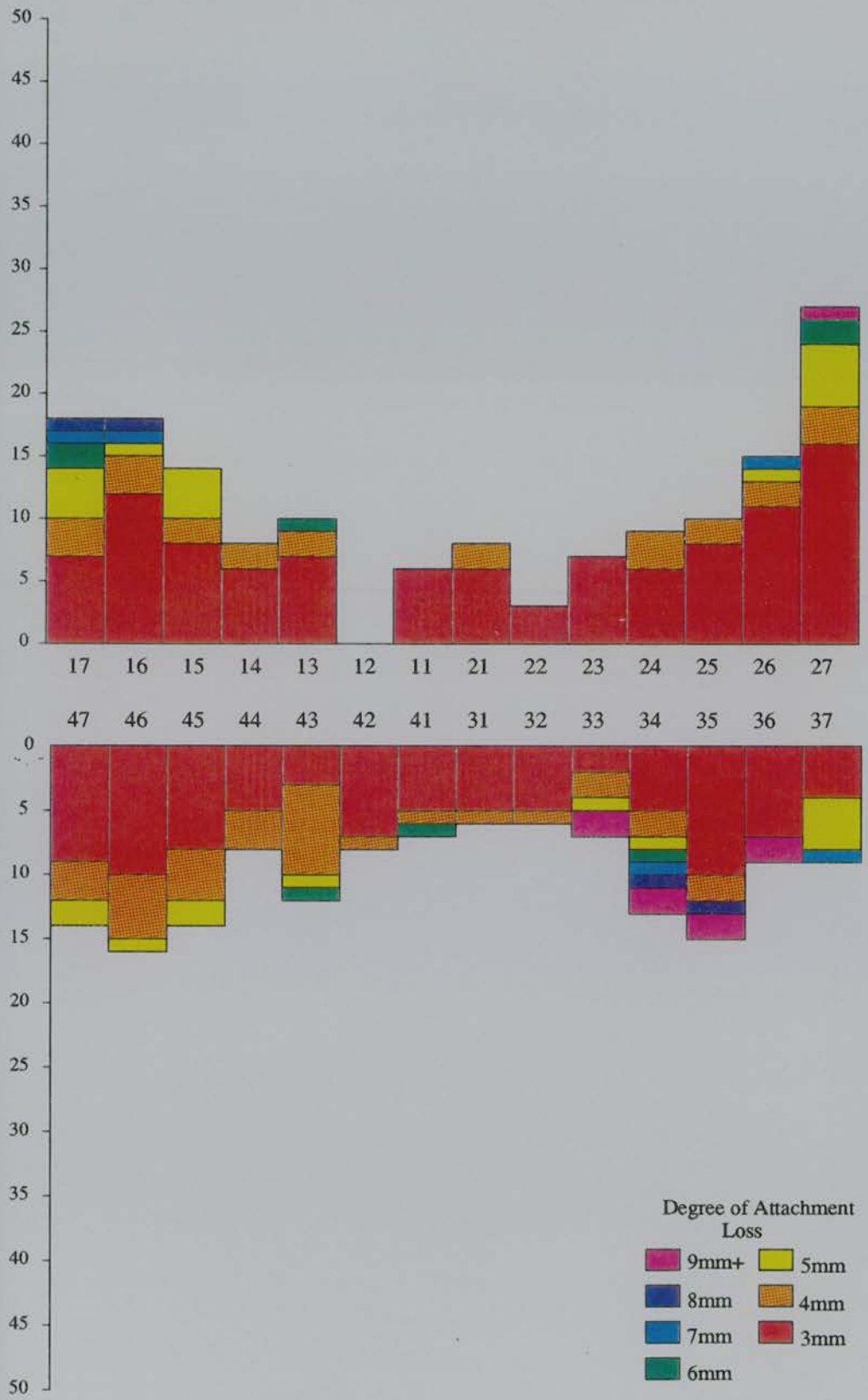


Figure 16 Mean Attachment Loss in HIV Seropositive and HIV Seronegative Subjects

Figures 17 and 18 plot the number of sites and their location within the mouth that had attachment loss of 3 mm or more at baseline examination in both HIV seronegative and HIV seropositive groups. The HIV seronegative group had 295 sites with attachment loss of 3 mm or more at the baseline visit, which represents 7.3% of all HIV seronegative sites examined at baseline (Figure 17). The HIV seropositive group yielded 818 sites with this degree of attachment loss or greater, which represented 18.2% of all sites of this group at baseline (Figure 18).



ıre 17 Location of Sites with 3 mm or More of Attachment Loss for HIV Seronegative Subjects

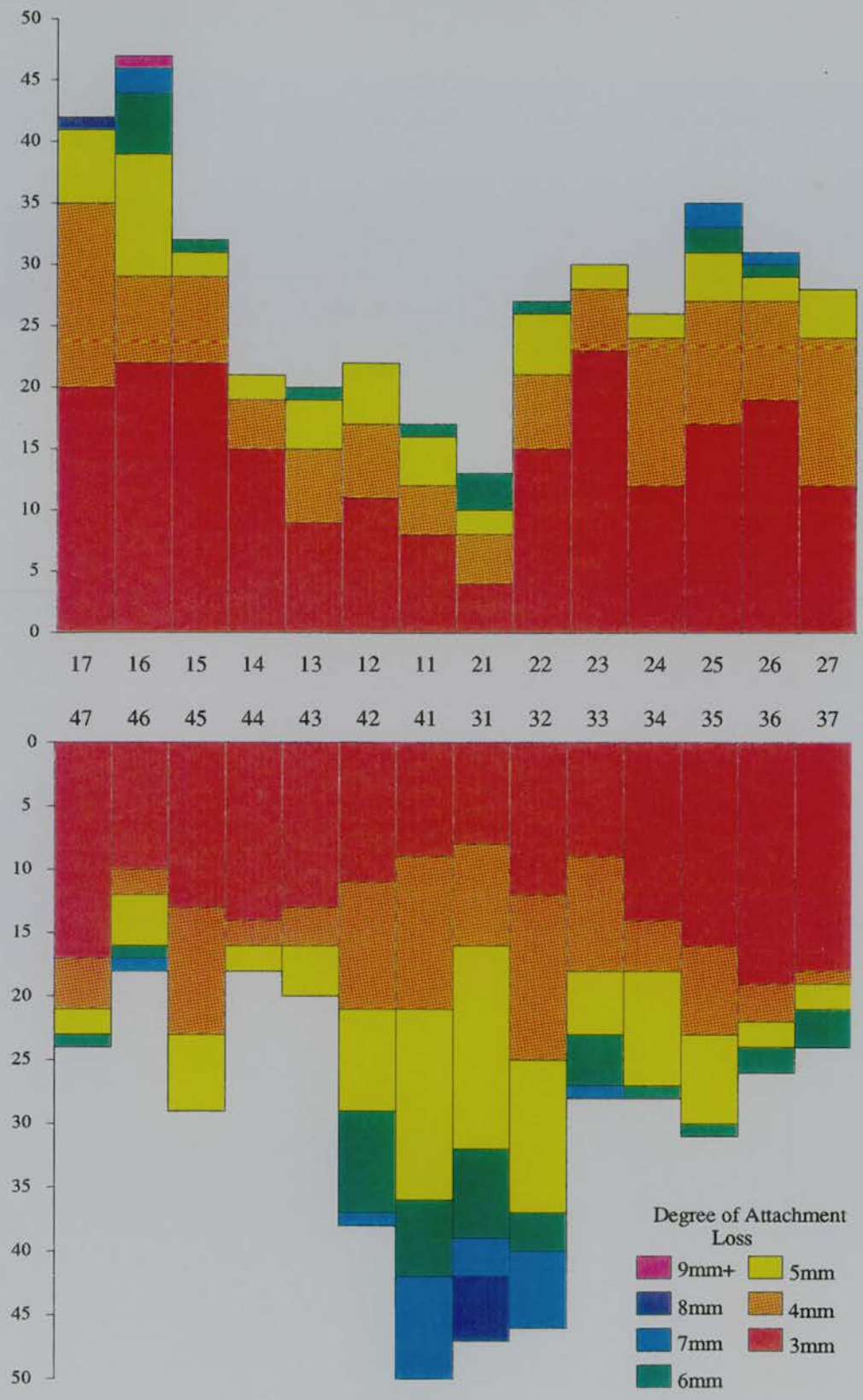


Figure 18 Location of Sites with 3 mm or More of Attachment Loss for HIV Seropositive Subjects

The pattern of attachment loss in the HIV seronegative group can be seen in Figure 17. Posterior teeth had increased prevalence and degree of attachment loss of 3 mm or more when compared to the anterior teeth for both upper and lower arches. Figure 18 shows the pattern of attachment loss in HIV seropositive patients as seen at baseline visit is different from that observed in HIV seronegative subjects. Whereas the pattern of loss in the upper arch was similar to that seen in the HIV seronegative group, the lower incisor teeth in HIV seropositive patients exhibited a markedly higher prevalence and degree of attachment loss when compared to other sites in the mouth. Furthermore, a large percentage of these sites had attachment loss of 5, 6 or 7 mm.

3.4.1.2 Widespread and localised periodontal disease

Patients were divided into those exhibiting widespread or localised disease on the basis of the percentage of sites with 3 mm or more of attachment loss (Haffajee *et al*, 1991). These authors decided that a patient would be categorised as having widespread periodontal disease if 30% or more of all sites had 3 mm or more of attachment loss. Localised periodontal disease patients would have less than 30% of all sites with this degree of attachment loss. According to these criteria, nine of 29 HIV seropositive patients and two of 27 HIV seronegative patients fell into the widespread disease category, with the remainder of both groups in the localised or no disease category. A chi-squared test was used to find if this difference in disease pattern between the groups was statistically significant (Table 15). Although showing a marked tendency, the difference between these two groups with respect to pattern of periodontal disease fell short of statistical significance ($p = 0.059$).

Table 15 Distribution of Widespread and Localised Periodontal Disease Categories Among HIV Seropositive and HIV Seronegative Subjects (Chi-square Test)

	HIV +	HIV -	p-value
Widespread	9	2	
Localised	20	25	0.059

The pattern of attachment loss of 3 mm or more in the nine HIV seropositive patients with widespread periodontal disease is shown in Figure 19, whereas Figure 20 plots the same for the remaining 20 HIV seropositive patients with localised or no disease. The nine HIV seropositive patients with widespread periodontal disease contributed a total of 636 sites with this degree of attachment loss, representing 48.2% of the total number of sites for these patients. The remaining 20 HIV seropositive patients had 182 sites with 3 mm or more of attachment loss, or 5.7% of the total number of sites for this group. The nine patients with widespread disease showed a marked tendency for moderate to severe attachment loss throughout the mouth, especially in the lower incisor region (Figure 19). Figure 20 shows that the 20 HIV seropositive patients with localised disease had a similar distribution to that seen in the HIV seronegative patient group as a whole (Figure 17). When mean attachment loss was compared between the widespread and localised periodontal disease patients of the HIV seropositive group, not surprisingly, the widespread patients had a statistically significantly higher mean attachment loss than localised periodontal disease patients ($p = 0.001$, Table 16). Eight of the widespread periodontal HIV seropositive patients were males, seven of whom were homosexuals or bisexuals and one IVDA. Further details of these patients are given in Table 17.

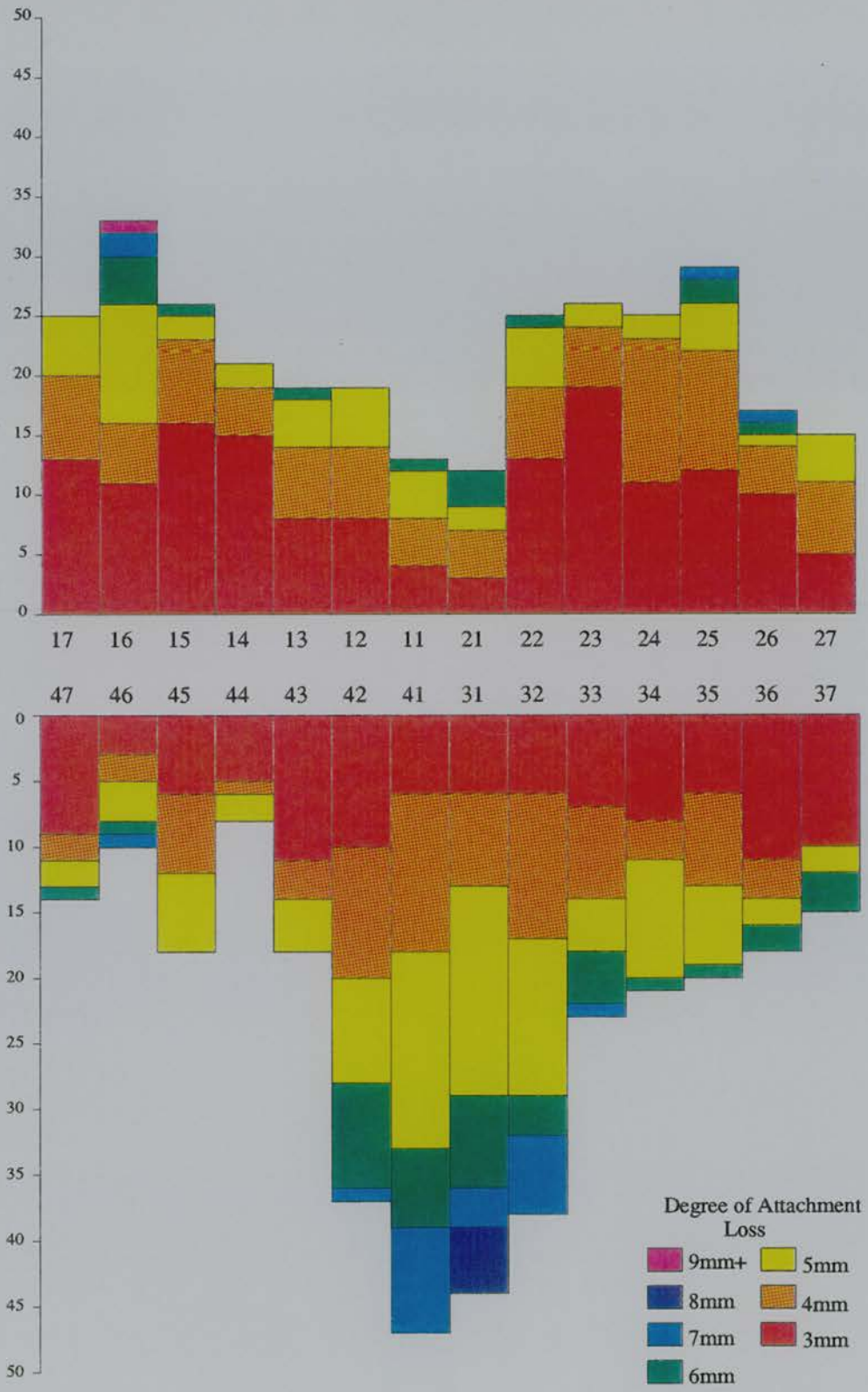


Figure 19 Location of Sites with 3 mm or More of Attachment Loss for Nine HIV Seropositive Subjects with Widespread Periodontal Disease

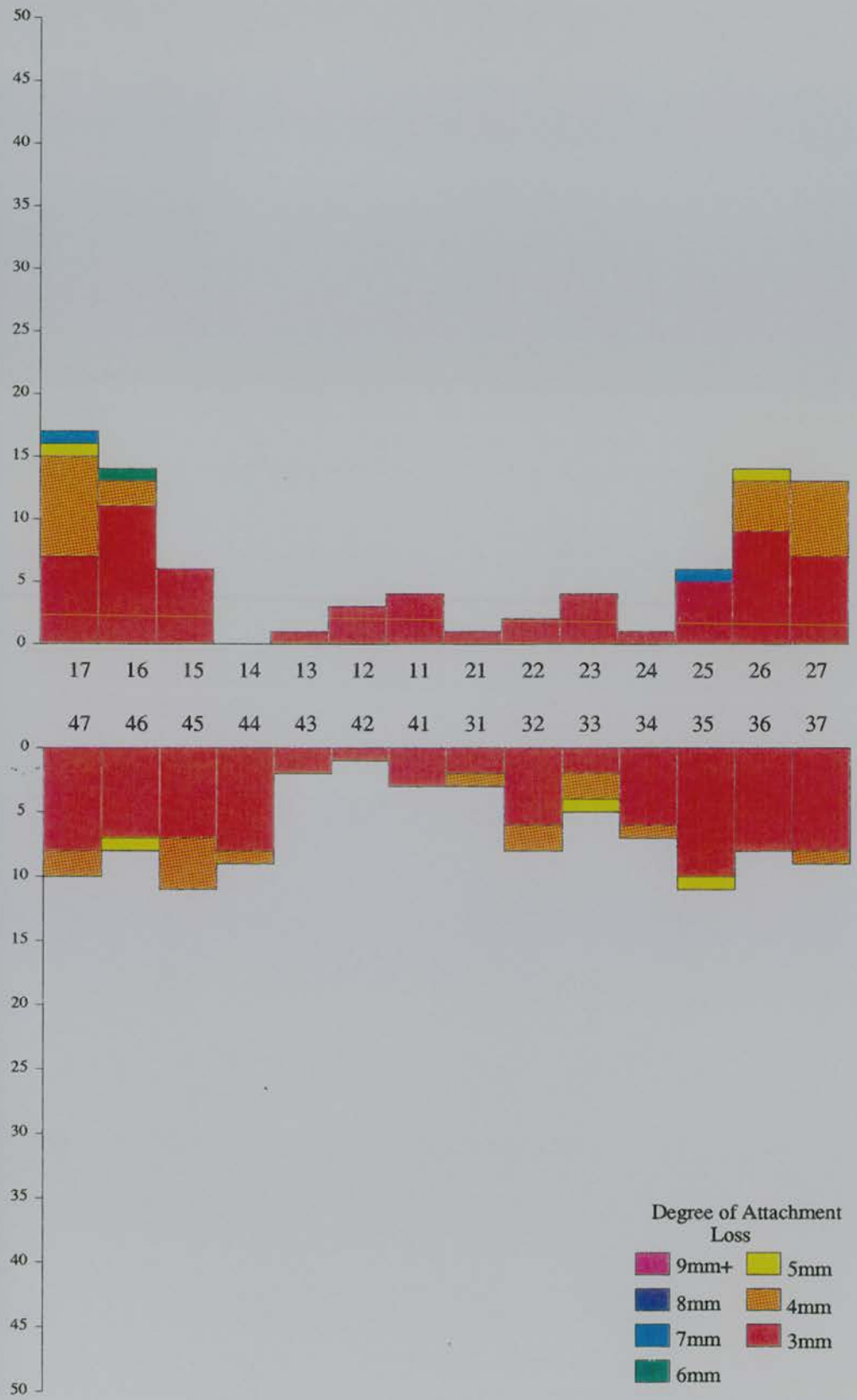


Figure 20 Location of Sites with 3 mm or More of Attachment Loss for 20 HIV Seropositive Subjects with Localised Periodontal Disease

Table 16 Comparison of Mean Attachment Loss of HIV Seropositive Subjects with Widespread and Localised Periodontal Disease (Mann-Whitney U Test)

	Median	Interquartile Range	p-value
Widespread	2.78	2.48 - 3.06	
Localised	0.97	0.523 - 1.57	0.001

Table 17 Details of HIV Seropositive Widespread and Localised Periodontal Disease Subjects

	widespread		localised			widespread		localised	
	n	%	n	%		n	%	n	%
male	8	89	15	75	CDC II	4	44	8	40
female	1	11	5	25	CDC IV	5	55	12	60
homo/bi	7	78	13	65	smoking	6	66	9	45
hetero	1	11	4	20	non smoking	3	33	11	55
IVDA	1	11	3	15					

Table 18 compares the mean attachment loss of 20 HIV seropositive and 25 HIV seronegative patients with localised periodontal disease. No difference of statistical significance could be found for mean attachment loss between these two groups.

Table 18 Comparison of Mean Attachment Loss of HIV Seropositive and HIV Seronegative Subjects with Localised Periodontal Disease (Mann-Whitney U Test)

	n	Median	Interquartile Range	p-value
HIV +	20	0.97	0.52 - 1.57	
HIV -	25	0.88	0.75 - 1.16	0.802

3.4.1.3 Dichotomous clinical indicators

Raw clinical data, including percentages of sites of individual patients with bleeding on probing, plaque, redness and suppuration, were not normally distributed for either HIV seropositive or HIV seronegative groups. To compare the presence or absence of any of these clinical indicators of periodontal disease across HIV seropositive and HIV seronegative groups, a Mann-Whitney U test was used (Table 19). No significant differences were found between these two groups with respect to bleeding on probing, plaque or redness. However, HIV seropositive patients had a statistically significantly higher percentage of sites exhibiting suppuration at baseline than HIV seronegative patients ($p = 0.025$).

Table 19 Comparison of Presence of Clinical Indicators Between HIV Seropositive and HIV Seronegative Subjects

(Mann-Whitney Test)

	Status	Median	Interquartile Range	p-value
% BOP	+	10.70	3.75 - 25.30	
	-	8.30	5.10 - 52.40	0.446
% PLQ	+	60.30	41.55 - 78.60	
	-	60.00	33.30 - 67.90	0.350
% RED	+	59.30	43.40 - 90.70	
	-	54.20	34.00 - 75.30	0.412
% SUPP	+	2.60	0.00 - 8.30	
	-	0.00	0.00 - 1.40	0.025

% BOP = percentage of sites exhibiting bleeding on probing

% PLQ = percentage of sites with plaque

% RED = percentage of sites with redness

% SUPP = percentage of sites with suppuration

In an attempt to investigate the nine widespread periodontal disease patients further, a Mann-Whitney U test was used to compare the incidence of the dichotomous clinical indicators between HIV seropositive patients with widespread and localised periodontal disease (Table 20). Although no differences of statistical significance were found between the percentage of sites with bleeding on probing or plaque between these two groups, the widespread periodontal disease group exhibited a statistically significantly higher percentage of sites with redness ($p = 0.030$) and suppuration ($p = 0.010$) than the HIV seropositive patients with localised periodontal disease.

Table 20 Comparison of Presence of Clinical Indicators in HIV Seropositve Subjects with Widespread and Localised Periodontal Disease (Mann-Whitney U Test)

	Disease	Median	Interquartile Range	p-value
% BOP	wide	13.70	7.60 - 17.35	
	local	9.05	1.65 - 39.18	0.396
% PLQ	wide	75.00	46.45 - 92.85	
	local	51.85	41.48 - 74.63	0.195
% RED	wide	96.70	41.35 - 100.00	
	local	53.45	42.25 - 70.73	0.030
% SUPP	wide	13.60	3.00 - 19.05	
	local	0.90	0.00 - 4.30	0.010

% BOP = percentage of sites exhibiting bleeding on probing

% PLQ = percentage of sites with plaque

% RED = percentage of sites with redness

% SUPP = percentage of sites with suppuration

Interestingly, no differences of statistical significance were found with respect to the dichotomous clinical indicators recorded for the 20 HIV seropositive and 25 HIV seronegative subjects with localised periodontal disease (Table 21).

Table 21 Comparison of Presence of Clinical Indicators in HIV Seropositive and HIV Seronegative Subjects with Localised Periodontal Disease (Mann-Whitney U Test)

	Status	Median	Interquartile Range	p-value
% BOP	+	9.05	1.65 - 39.18	
	-	7.70	4.70 - 51.20	0.293
% PLQ	+	51.85	41.48 - 74.63	
	-	56.90	32.05 - 67.75	0.640
% RED	+	53.45	42.25 - 70.73	
	-	53.60	33.35 - 75.70	0.936
% SUPP	+	0.90	0.00 - 4.30	
	-	0.00	0.00 - 1.30	0.158

3.4.2 Subsequent Visits

Eighteen HIV seropositive patients were seen at a second visit, and nine of these returned for at least a third visit (Tables 22 and 23). Wilcoxon matched pairs signed ranks test was used to compare the changes in attachment loss and pocket depth as well as changes in the percentage of sites with clinical indicators of periodontal disease of each patient between baseline and visit two and between visits two and three (Tables 22 and 23).

Table 22 Comparison of Attachment Loss and Pocket Depth for HIV Seropositive Subjects Returning on Subsequent Visits
(Wilcoxon Matched Pairs Signed Ranks Test)

	n	Median	Interquartile Range	p-value
pocket depth				
visit 1	18	2.23	1.72 - 2.75	
visit 2	18	2.10	1.69 - 2.39	0.011
visit 2	9	2.23	1.87 - 2.62	
visit 3	9	2.06	1.65 - 2.67	0.139
attachment loss				
visit 1	18	1.71	0.80 - 2.38	
visit 2	18	1.40	0.97 - 2.36	0.118
visit 2	9	1.97	1.15 - 2.44	
visit 3	9	1.91	0.86 - 2.42	0.066

Table 23 Comparison of Clinical Indicators for HIV Seropositive Subjects Returning on Subsequent Visits

(Wilcoxon Matched Pairs Signed Ranks Test)

	n	Median	Interquartile range	p-value
% BOP				
visit 1	18	15.10	6.13 - 42.73	
visit 2	18	11.05	3.55 - 21.48	0.001
visit 2	9	8.30	3.05 - 20.25	
visit 3	9	10.80	1.65 - 18.8	0.767
% PLQ				
visit 1	18	53.95	36.30 - 76.18	
visit 2	18	67.30	46.88 - 74.10	0.055
visit 2	9	65.50	55.65 - 75.65	
visit 3	9	66.70	39.00 - 82.60	0.441
% RED				
visit 1	18	57.09	46.88 - 82.53	
visit 2	18	73.45	58.48 - 94.45	0.124
visit 2	9	62.30	52.65 - 95.80	
visit 3	9	75.00	44.40 - 85.50	0.779
% SUPP				
visit 1	18	3.45	0.45 - 9.85	
visit 2	18	2.05	0.00 - 8.15	0.856
visit 2	9	6.50	0.60 - 9.15	
visit 3	9	1.90	0.00 - 11.25	0.441

3.4.2.1 Attachment loss and pocket depth

Mean pocket depth decreased in 14 HIV seropositive patients between baseline and visit two ($p = 0.011$). No statistically significant differences were observed for mean pocket depth between visit two and three. There was a tendency for the mean attachment level to improve between visits two and three in seven of the nine HIV seropositive patients but this was not statistically significant ($p = 0.066$, Table 22).

3.4.2.2 Dichotomous clinical indicators

Of a total of 18 HIV seropositive patients seen at visit two, 16 had fewer sites with bleeding on probing on the second visit compared to the baseline examination ($p = 0.001$), but a tendency for increased plaque on the second visit was seen in 13 patients ($p = 0.055$). There were no statistically significant differences in the percentages of sites with redness or suppuration between these two visits. There were no statistically significant differences between the percentages of the various clinical indicators of the nine HIV seropositive patients between visits two and three (Table 23).

3.5 MICROBIOLOGICAL DATA

The baseline microbiological data reported below is for 20 HIV seropositive patients and 23 HIV seronegative patients. This represents 445 sites in the HIV seropositive patients and 497 sites in the HIV seronegative patients or 86% of the total number of sites for both groups. Tables 24 and 25 give the details of these 43 patients.

Table 24 Microbiology Data Patient Statistics 1

	HIV +			HIV -		
	n	AGE	sd	n	AGE	sd
Male	16	37.0	10.9	15	34.5	9.6
Female	4	26.0	3.6	8	38.3	15.5
Total	20	34.8	10.8	23	35.9	11.8

Table 25 Microbiology Data Patient Statistics 2

(HIV + only)

	n	%		n	%
homo/bi	14	70	CDC II	11	55
hetero	4	20	CDC IV	9	45
IVDA	2	10	smoking	11	55
			non smoking	9	45

3.5.1 Lower limit of detection

The lower limit of detection of the colony lift method was dependent on the total colony count for each site and therefore varies from site to site within a patient. The mean total colony count for the HIV seropositive sites was 651.5 (s.d. 218.00) compared to 657.3 (s.d. 221.32) for HIV seronegative sites. The mean lower limit of detection of colony lifts prepared from HIV seropositive sites was 0.58% (s.d. 0.18) compared to a mean lower limit of detection of 0.60% (s.d. 0.25) in sites from HIV seronegative patients.

3.5.2 Prevalence

Table 26 gives the percentage of patients in both groups positive for the species enumerated. *V. parvula* and *W. recta* were detected in at least one site in all 43 patients, whereas *P. gingivalis* was detected in 18 of the 20 HIV seropositive patients and 19 of 23 HIV seronegative patients. Included in the table are the results of the study by Haffajee *et al* (1992) of adult/chronic periodontitis patients for comparison. The percentage of sites positive for each of the species enumerated is given in Table 27. *V. parvula* was detected in 50.1% of the HIV seropositive sites and 55.3% of the sites from HIV seronegative patients. This table also includes data from Gunaratnam *et al* (1992) for percentage of sites positive for these strains in 90 periodontitis patients whose HIV status was unknown.

Table 26 Percentage of Subjects Positive for Probe Species Enumerated

	HIV +	HIV -	Haffajee *et al*, (1992)
A. actinomycetemcomitans	80	91	52
P. gingivalis	90	83	91
P. intermedia	85	96	98
C. rectus	100	100	83
B. forsythus	100	91	76
C. ochracea	75	91	94
V. parvula	100	100	98
Number of Subjects	20	23	90

Table 27 Percentage of Sites Positive for Probe Species Enumerated

	HIV +	HIV -	Gunaratnam *et al*, (1992)
A. actinomycetemcomitans	25.8	27.0	13.0
P. gingivalis	32.4	18.3	37.0
P. intermedia	24.3	27.2	36.0
C. rectus	38.4	41.0	48.0
B. forsythus	32.8	36.8	22.0
C. ochracea	24.3	25.2	42.0
V. parvula	50.1	55.3	59.0
Number of Sites	445	497	700

3.5.3 Comparison Between HIV Seropositive and HIV Seronegative Subjects

A Mann-Whitney U test was used to compare the difference between HIV seropositive and HIV seronegative patients on the basis of mean percentage of the species enumerated. Table 28 gives the median for each species for both groups as well as the interquartile range and the p-value for the Mann-Whitney U test for each species. No differences of statistical significance were found between groups with regard to the mean percentage of six of the seven species. However, *P. gingivalis* was found at a higher mean percentage in the HIV seropositive patients compared to the HIV seronegative controls ($p = 0.030$).

Table 28 Comparison of Mean Percentages of Probe Species Enumerated in HIV Seropositive and HIV Seronegative Subjects

(Mann-Whitney U Test)

	Status	Median	Interquartile Range	p-value
A. actinomycetemcomitans	+	0.21	0.04 - 1.99	
	-	0.53	0.19 - 0.87	0.798
P. gingivalis	+	0.73	0.28 - 1.58	
	-	0.22	0.02 - 0.72	0.030
P. intermedia	+	0.44	0.07 - 1.03	
	-	0.42	0.09 - 1.08	0.807
C. rectus	+	0.65	0.26 - 1.91	
	-	0.86	0.61 - 2.03	0.233
B. forsythus	+	1.06	0.47 - 1.68	
	-	0.96	0.38 - 2.11	0.855
C. ochracea	+	0.35	0.02 - 1.15	
	-	0.59	0.12 - 0.97	0.788
V. parvula	+	2.08	0.64 - 5.53	
	-	1.86	1.23 - 3.59	0.865
Total	+	11.21	6.37 - 19.13	
	-	9.79	5.81 - 13.25	0.295

3.5.4 Widespread and Localised Disease in HIV Seropositive Subjects

Microbiological data were available for only seven of the nine HIV seropositive patients with widespread periodontal disease. These patients were compared to the remaining 13 patients with localised or no disease. Widespread periodontal disease patients exhibited a higher mean percentage of these seven probe species than the HIV seronegative patients. This difference was statistically significant ($p = 0.005$, Table 29). The HIV seropositive patients with widespread periodontal disease had a median mean total of 19.18% compared to the localised disease group with a median mean total of 9.34% (Table 29). Furthermore, these seven HIV seropositive patients also had a tendency for a higher mean percentage of *P. gingivalis* ($p = 0.063$), *P. intermedia* ($p = 0.088$), *C. ochracea* ($p = 0.066$) and *V. parvula* ($p = 0.081$) (Table 29). No pattern of colonisation of any one of the probe species could be associated with the lower incisor region of the seven widespread disease patients.

Table 29 Comparison of Mean Percentages of Probe Species Enumerated in HIV Seropositive Subjects with Widespread or Localised Periodontal Disease (Mann-Whitney U Test)

	Disease	Median	Interquartile Range	p-value
A. actinomycetemcomitans	wide	2.09	0.05 - 3.41	
	local	0.12	0.02 - 0.95	0.111
P. gingivalis	wide	1.38	0.68 - 1.68	
	local	0.43	0.21 - 1.00	0.063
P. intermedia	wide	0.45	0.28 - 1.71	
	local	0.24	0.02 - 0.76	0.088
C. rectus	wide	1.39	0.14 - 2.04	
	local	0.62	0.30 - 2.70	0.843
B. forsythus	wide	1.40	0.90 - 3.36	
	local	0.88	0.43 - 1.30	0.166
C. ochracea	wide	1.21	0.10 - 4.53	
	local	0.29	0.00 - 0.66	0.066
V. parvula	wide	3.87	1.32 - 11.90	
	local	1.32	0.43 - 4.53	0.081
Total	wide	19.18	12.95 - 24.51	
	local	9.34	4.86 - 14.15	0.005

HIV+ widespread = 7, localised = 13.

3.5.5 HIV Seropositive Subjects and HIV Seronegative Subjects with Localised Periodontal Disease

Table 30 compares the mean percentages of the species enumerated in the localised or no disease group of 13 HIV seropositive and 21 HIV seronegative subjects. Using a Mann-Whitney U test no differences of statistical significance could be found between these two groups with respect to any of the species enumerated.

Table 30 Comparison of Mean Percentages of Probe Species Enumerated in HIV Seropositive and HIV Seronegative Subjects with Localised Periodontal Disease (Mann-Whitney U Test)

	Status	Median	Interquartile Range	p-value
A. actinomycetemcomitans	+	0.12	0.02 - 0.95	
	-	0.53	0.19 - 0.85	0.166
P. gingivalis	+	0.43	0.21 - 1.00	
	-	0.31	0.03 - 0.79	0.425
P. intermedia	+	0.24	0.02 - 0.76	
	-	0.52	0.10 - 1.19	0.178
C. rectus	+	0.62	0.30 - 2.70	
	-	0.86	0.59 - 1.93	0.215
B. forsythus	+	0.88	0.43 - 1.30	
	-	0.96	0.34 - 2.26	0.632
C. ochracea	+	0.29	0.00 - 0.66	
	-	0.65	0.13 - 1.10	0.15
V. parvula	+	1.32	0.43 - 4.53	
	-	1.83	1.17 - 3.49	0.512
Total	+	9.34	4.86 - 14.15	
	-	9.79	6.00 - 13.39	0.583

3.6 IMMUNOLOGY DATA

Data on a number of immunological markers were available for 28 of 29 HIV seropositive patients, including $CD4^+$ and $CD8^+$ counts, activated T-cells and β_2-microglobulin. However, the date of baseline periodontal examination did not always coincide with the date of the immunology results. Six HIV seropositive patients had immunological results within one week of the baseline examination and twelve patients had results, both before and after baseline examination within three months of this visit. Four patients had a single immunology result within three months either before or after baseline visit and five patients had no immunology results within three months of baseline visit. Immunology results could not be traced for two HIV seropositive patients. Only $CD4^+$ and $CD8^+$ T-cell counts were used for statistical analyses because these were the most frequently performed immunological tests, whereas β_2-microglobulin, activated T-cells and immunoglobulin assays were performed only occasionally.

3.6.1 Levels of $CD4^+$ and $CD8^+$ T-cells

In an attempt to provide an accurate estimate of the levels of $CD4^+$ and $CD8^+$ T-cells at baseline visit the following regime was adopted. If the immunology results were within one week of baseline, then this single value of $CD4^+$ or $CD8^+$ T-cells was taken as an estimate. If either one or both results from before or after the baseline visit was within three months, then an average of both results was taken as an estimate of levels at baseline. If more than three months separated the baseline examination and the immunology results, then these patients were excluded from the analysis. A total of 22 HIV seropositive patients satisfied the above criteria and details of these patients are given in Tables 31 and 32. Median and interquartile range of $CD4^+$ and $CD8^+$ T-cell counts for these patients and subgroups of CDC stage II and IV are given in Table 33.

As would be expected the CDC stage II patients have higher counts of $CD4^+$ and $CD8^+$ T-cells than the CDC stage IV patients. The CDC stage IV group has a lower $CD4^+$ T-cell count and $CD4^+$: $CD8^+$ ratio than the CDC stage II group ($p = 0.006$ and $p = 0.014$ respectively). The CDC stage IV group had also a tendency for a lower $CD8^+$ T-cell count ($p = 0.070$).

Table 31 Immunology Data Patient Statistics 1

	n	HIV + AGE	sd
male	19	36.2	8.65
female	3	27.3	3.21
Total	22	35.0	8.64

Table 32 Immunology Data Patient Statistics 2

(HIV+ only)

	n	%		n	%
homo/bi	17	77.2	CDC II	8	36.4
hetero	2	9.1	CDC IV	14	63.6
IVDA	3	13.6	smoking	11	50
			non smoking	11	50

Table 33 CDC Stage and CD4, CD8 T-Cell Counts and CD4 : CD8 Ratio

(Mann-Whitney U Test)

Marker		Median	Interquartile range	p-value
CD4	normal	500 - 750		
	CDC II	252.50	180.75 - 569.00	
	CDC IV	70.00	6.50 - 194.50	0.006
CD8	normal	750 - 1000		
	CDC II	910.00	464.70 - 1240.00	
	CDC IV	560.00	145.00 - 915.00	0.070
CD4 : CD8	normal	2.0		
	CDC II	0.332	0.20 - 0.59	
	CDC IV	0.091	0.03 - 0.32	0.014

3.6.2 Attachment Loss

No correlation was found between the mean attachment loss in 22 HIV seropositive patients at baseline visit and $CD4^+$, $CD8^+$ T-cell counts and $CD4^+$: $CD8^+$ ratio.

3.6.3 Widespread and Localised Disease

Immunological results were available for seven of the nine HIV seropositive patients with widespread periodontal disease, the remaining 15 HIV seropositive patients with immunological results having localised periodontal disease. Table 34 compares the levels of $CD4^+$, $CD8^+$ T-cell counts and $CD4^+$: $CD8^+$ ratio for these two groups using a Mann-Whitney U test. No statistically significant differences in $CD4^+$, $CD8^+$ T-cell count or $CD4^+$: $CD8^+$ ratio were found between these two groups (Table 34).

Table 34 Comparison of CD4, CD8 T-Cell Counts and CD4 : CD8 Ratio in HIV Seropositive Subjects with Widespread and Localised Periodontal Disease (Mann-Whitney U Test)

Marker	Disease	Median	Interquartile Range	p-value
CD4	wide	175.00	3.00 - 247.00	
	local	159.00	37.00 - 240.00	0.526
CD8	wide	471.00	109.00 - 1160.00	
	local	660.00	450.00 - 968.00	0.307
CD4 : CD8	wide	0.13	0.03 - 0.38	
	local	0.19	0.08 - 0.54	0.438

3.6.4 Dichotomous Clinical Indicators

Plots of $CD4^+$, $CD8^+$ T-cells and $CD4^+$: $CD8^+$ ratio against clinical indicators for the 22 HIV seropositive patients showed no correlation between these immunological markers and clinical indicators, with the possible exception of redness. Whereas $CD4^+$ and $CD8^+$ counts, and percentage of sites with redness did not show any correlation, the $CD4^+$: $CD8^+$ ratio tended to be weakly negatively correlated with the percentage of sites with redness ($R = -0.362$, $p = 0.098$, Figure 21).

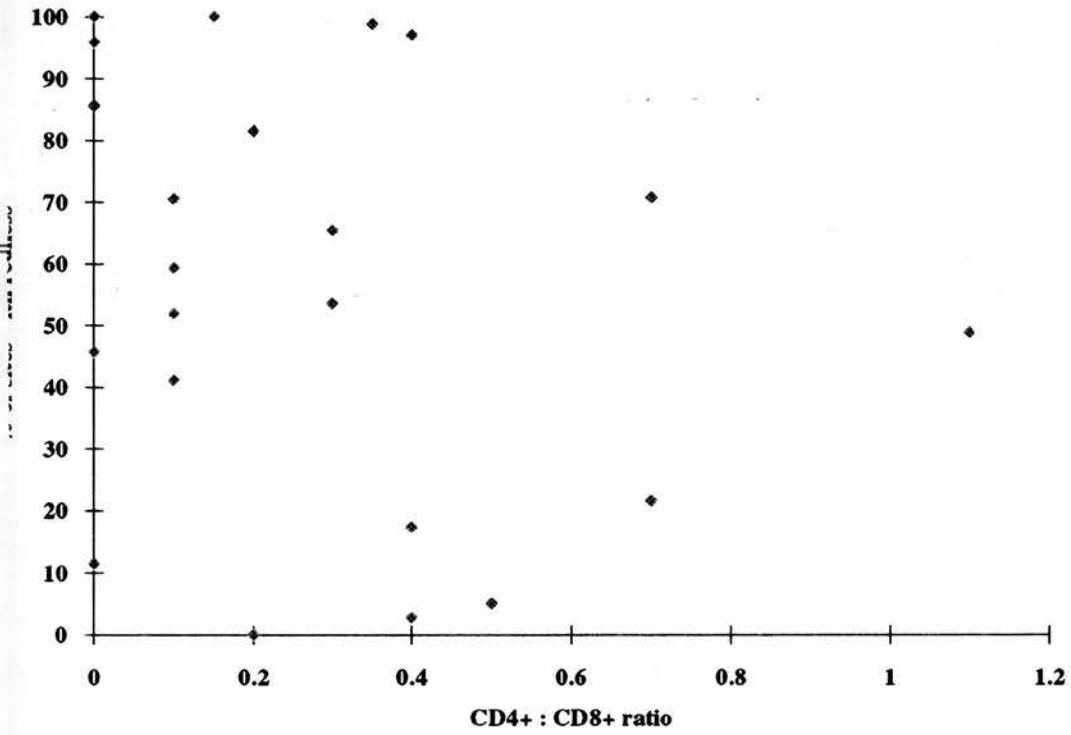


Figure 21 Plot of CD4+ : CD8+ Ratio Against Percentage of Sites with Redness in HIV Seropositive Subjects

3.6.5 Microbiology

No correlation was found between mean levels of any of the probe species and $CD4^+$, $CD8^+$ T-cells counts or $CD4^+$: $CD8^+$ ratio.

3.7 ACTIVE AND CONTROL SITES

Seven HIV seropositive patients were identified as having at least one site that experienced an increase in attachment loss of 3 mm or more between subsequent visits. Two patients were judged to have attachment loss of this degree at different sites on two separate occasions. Table 35 details the number of active and control sites sampled between visits two and four, along with the number of patients contributing these sites. Seven sites were identified from 2814 reviewed at the second visit to have increased in attachment loss by 3 mm or more since baseline examination. Similarly, four sites from 1404 were identified at visit three and nine sites identified from 792 reviewed at visit four. All of these sites came from homosexual or bisexual males with five of these subjects CDC stage IV and two CDC stage II. Further details of these patients are given in Table 36. Results of $CD4^+$, $CD8^+$ T-cell counts and ratio for these seven patients are given in Table 37. All patients experiencing breakdown tended to have low $CD4^+$ T-cell counts.

Table 35 Active and Control Sites

	Visit 2	3	4	5	6
Number of patients reviewed	18	9	5	3	1
Number of sites reviewed	2814	1404	792	489	168
Number of patients with active sites	4	3	2*	0	0
Active sites Sampled	7	4	9	0	0
Control sites Sampled	4	4	2	0	0
Rate of occurence of active sites	0.25%	0.29%	1.1%	-	-

(*both patients had previous occurence of active sites)

Table 36 Patient Statistics of HIV Seropositive Subjects with Active Sites

	n	%		n	%
homo/bi	7	100	CDC II	2	28.6
hetero	-	0	CDC IV	5	71.4
IVDA	-	0			
widespread	4	57.1	smoking	2	28.6
localised	3	42.9	non smoking	5	71.4

Table 37 CD4, CD8 T-Cell Counts and CD4 : CD8 Ratio in HIV Seropositive Subjects with Active Sites

Marker	Median	Interquartile Range
CD4	153.50	27.25 - 268.50
CD8	543.00	210.00 - 889.75
CD4 : CD8	0.22	0.07 - 0.33

3.7.1 Clinical Data from Active and Control Sites

Tables 38 and 39 give the mean attachment loss and percentages of clinical indicators at both active and control sites. The mean attachment level of active sites was 5.15 mm (s.d 1.76) compared to 4.90 mm (s.d. 2.02) for control sites (Table 38). A Chi-square test was used to compare the presence of these clinical indicators with the clinical classification of active or control sites (Table 39). There was no statistically significant differences between active and control sites with regard to dichotomous clinical indicators, although 35% of active sites exhibited bleeding on probing compared to only 10% of control sites. Similarly, 70% of active sites had redness, whereas only 40% of control sites exhibited redness.

Table 38 Mean Attachment Level Loss of Active and Control Sites

	Active	Control
Number of Sites	20	10
Mean Attachment Level Loss	5.15	4.90
(sd)	1.76	2.02

Table 39 Comparison of Clinical Indicators Across Active and Control Sites (Chi-square Tests)

	Active	Control	p-value
Number of sites	20	10	
BOP (n)	7	1	0.307
% BOP	35	10	
PLQ (n)	14	7	1.000
% PLQ	70.0	70.0	
RED (n)	14	4	0.236
% RED	70.0	40.0	
SUPP (n)	3	0	0.519
% SUPP	15.0	0.0	

3.7.2 Comparison of Microbiology of Active and Control Sites

To compare the microbiology of active and control sites a Mann-Whitney U test was used. Table 40 details the median, interquartile range and p-value for the statistical test as before for the probe species across active and control sites. *C. ochracea* was not detected in either active or control sites. Of the remaining species no statistically significant differences in the levels of any of the probe species were found between active and control sites (Table 40).

Table 40 Comparison of Microbiology of Active and Control Sites

(Mann-Whitney)

	Condition	Median	Interquartile Range	p-value
A. actinomycetemcomitans	active	0.00	0.00 - 0.60	
	control	0.00	0.00 - 1.25	0.408
P. gingivalis	active	0.50	0.00 - 8.80	
	control	0.00	0.00 - 1.23	0.265
P. intermedia	active	0.80	0.00 - 3.40	
	control	1.40	0.10 - 4.30	0.409
C. rectus	active	0.50	0.00 - 2.80	
	control	0.60	0.00 - 1.53	0.912
B. forsythus	active	0.00	0.00 - 1.60	
	control	0.30	0.00 - 4.63	0.540
V. parvula	active	2.70	0.00 - 5.80	
	control	0.25	0.00 - 9.68	0.611
Total	active	8.00	6.00 - 18.30	
	control	7.75	3.98 - 21.75	0.710

(*C. ochracea* was not isolated from either active or control sites)

3.8 PERIODONTAL PARAMETERS AS POSSIBLE PREDICTORS OF HIV DISEASE PROGRESSION

In an attempt to provide some information regarding possible predictors of HIV disease progression, cross-sectional clinical and microbiological data were used in conjunction with a Kruskal - Wallis test, (one way analysis of variance for non-parametric data) across the following groups, HIV seronegative, CDC stage II, CDC stage IV.

3.8.1 Attachment Loss

Table 41 shows that while there was no difference between the HIV seronegative group and the CDC stage II HIV seropositive patients with regard to mean attachment loss, CDC stage IV patients had a statistically significantly higher mean attachment loss than any of the other groups ($p = 0.036$).

Table 41 Comparison of Mean Attachment Loss Across HIV Disease Status (Kruskal-Wallis Test)

	HIV -	CDC II	CDC IV	p-value
median	0.90	0.68	1.65	
i.q. range	0.77 - 1.38	0.49 - 2.63	1.13 - 2.47	
mean rank	24.78	24.83	37.00	0.036

i.q. range = interquartile range

3.8.2 Dichotomous Clinical Indicators

Table 42 details the differences in dichotomous clinical indicators between the three groups. The percentage of sites exhibiting suppuration is seen to increase across the three CDC groups, with HIV seronegative subjects having the lowest mean percentages and the CDC stage IV patients having the highest mean percentages of sites exhibiting suppuration. These differences were found to be statistically significant ($p = 0.026$). No other differences of statistical significance were detected.

Table 42 Comparison of Clinical Indicators Across HIV Disease Status

(Kruskal-Wallis Test)

	HIV -	CDC II	CDC IV	p-value
mean % BOP	8.30	7.95	11.30	
i.q. range	5.10 - 52.40	0.83 - 26.85	4.80 - 31.55	
mean rank	30.22	23.92	29.00	0.531
mean % PLQ	60.00	57.25	63.60	
i.q. range	33.30 - 67.90	46.40 - 83.98	35.80 - 76.85	
mean rank	26.39	32.04	29.35	0.587
mean % RED	54.20	67.30	56.50	
i.q. range	34.00 - 75.30	28.40 - 92.9	43.40 - 90.70	
mean rank	26.65	30.42	30.09	0.714
mean % SUPP	0.00	0.30	4.30	
i.q. range	0.00 - 1.40	0.00 - 5.15	0.30 - 10.80	
mean rank	23.69	27.83	36.62	0.026

3.8.3 Microbiological Data

Differences in mean percentage of the probe species enumerated across the three groups are given in Table 43. The mean percentage of *P. gingivalis* displayed a tendency to increase throughout the groups ($p = 0.055$), with HIV seronegative subjects having on average lower mean percentages of *P. gingivalis* than CDC stage II HIV seropositive patients, who have in turn on average lower mean percentages of *P. gingivalis* than CDC stage IV patients. The mean percentages of *V. parvula* were highest for CDC stage II patients but had dropped to very low levels in CDC stage IV patients ($p = 0.037$). No other differences of statistical significance were found between these groups with respect to levels of species enumerated.

Table 43 Comparison of Mean Percentage of Probe Species Enumerated Across HIV Disease Status (Kruskal-Wallis Test)

	HIV -	CDC II	CDC IV	p-value
mean % Aa	0.53	0.15	0.52	
i.q. range	0.19 - 0.87	0.04 - 1.56	0.03 - 3.43	
mean rank	22.46	19.30	23.65	0.716
mean % Pg	0.22	0.39	0.87	
i.q. range	0.02 - 0.72	0.19 - 1.66	0.58 - 1.45	
mean rank	18.13	23.50	29.40	0.055
mean % Pi	0.42	0.27	0.76	
i.q. range	0.09 - 1.08	0.00 - 0.54	0.20 - 1.29	
mean rank	22.43	16.50	26.50	0.198
mean % Cr	0.86	0.52	0.68	
i.q. range	0.61 - 2.03	0.21 - 1.65	0.31 - 2.54	
mean rank	24.13	18.35	20.75	0.448
mean % Bf	0.96	1.11	0.97	
i.q. range	0.38 - 2.11	0.11 - 5.40	0.55 - 1.35	
mean rank	21.67	23.10	21.65	0.951
mean % Co	0.59	0.18	0.92	
i.q. range	0.12 - 0.97	0.00 - 0.54	0.26 - 2.27	
mean rank	22.48	16.25	26.65	0.172
mean % Vp	1.86	4.63	1.11	
i.q. range	1.23 - 3.59	1.77 - 8.60	0.44 - 2.18	
mean rank	21.70	29.55	15.15	0.037
mean % Total	9.79	11.21	11.64	
i.q. range	5.81 - 13.25	8.60 - 21.05	5.25 - 19.02	
mean rank	20.13	25.50	22.80	0.515

i.q. range = interquartile range

CHAPTER FOUR

DISCUSSION

4.1 OBJECTIVES OF STUDY

The objectives set out at the beginning of this thesis were:

1) To establish a procedure for the enumeration of selected periodontal bacteria in a large number of plaque samples using non-isotopic whole chromosomal DNA probes.

2) To compare the prevalence of periodontal disease between HIV seropositive and HIV seronegative subjects in the Edinburgh area.

3) To compare the prevalence of these selected periodontal bacteria between HIV seropositive and HIV seronegative patients.

4) To assess the incidence of HIV-associated periodontal diseases in the HIV seropositive group on a longitudinal basis.

5) To characterise the microbiology associated with these lesions in terms of those species selected for enumeration.

6) To investigate whether periodontal breakdown or any of the clinical or microbiological parameters studied could be used as predictors of HIV disease progression.

To discuss the fulfilment of the first objective, the advantages and disadvantages of using whole chromosomal probes to detect periodontal bacterial DNA on nylon colony lifts will be addressed, followed by a discussion of the modifications to the technique, as

originally described by Gunaratnam *et al* (1992), that were used in this study to improve efficiency in terms of labour, cost and time.

4.1.1 Whole Chromosomal DNA Probes

Traditional methods of identification of bacterial species in subgingival plaque samples include the predominant cultivable microbiota technique (PCM). While PCM provides, arguably, the widest picture of the subgingival microbiota (Dzink *et al*, 1985, 1988; Haffajee *et al*, 1988a,b; Moore *et al*, 1982; Tanner *et al*, 1979), subculture and identification to species level of up to 50 randomly chosen colonies on a primary isolation plate can take up to 6 - 8 weeks. Furthermore, it may result in inconclusive biochemical tests for some isolates after a considerable investment in time and labour. However, the development of digoxigenin-labelled whole chromosomal DNA probes, used in conjunction with colony lifts of primary isolation plates, enables a more rapid enumeration of a selection of periodontal species from a larger number of plaque samples than previously possible using PCM (Gunaratnam *et al*, 1992). Using this technique, enumeration of a selection of periodontal species in a subgingival plaque sample can be completed within ten days. Although several techniques involving nucleic acid probes are available for the identification of periodontal species in subgingival plaque samples (see Literature Review section 1.5), the use of whole chromosomal digoxigenin-labelled probes has several advantages.

The extraction of whole chromosomal DNA from bacteria is relatively straightforward and protocols exist for both Gram-negative and Gram-positive bacteria (Smith *et al*, 1989b,c). These protocols generally take in the region of 4 - 6 hours and yields of up to several hundred micrograms of whole chromosomal DNA are possible with some species. Due to their size, whole chromosomal probes generally incorporate a larger number of reporter molecules per probe than either cloned or oligonucleotide probes.

The number of reporter molecules per probe will depend on the labelling method used but can result in a stronger signal when hybrids are detected. Furthermore, the use of whole chromosomal DNA probes may be considered more sensitive than either oligonucleotide or cloned DNA probes because the entire genome of the species under investigation is targeted, whereas oligonucleotide or cloned DNA probes target only a small section of the DNA (Tenover, 1988; Albandar and Olsen, 1990). However, this feature of whole chromosomal DNA probes may result in cross-reactions between the DNA of closely related species. Gunaratnam *et al* (1992) reduced the occurrence of cross-reactions observed for a selection of periodontal species by using conditions of high stringency. These authors validated the following species *P. gingivalis, P. intermedia* (homology groups 1 and 2), *B. forsythus, A. actimomycetemcomitans* (serotypes a and b), *C rectus, V. parvula, C. ochracea, F. nucleatum, Streptococcus intermedius, Streptococcus sanguis I, Streptococcus sanguis II,* and *Peptostreptococcus micros* against 249 strains representing a total of 51 species. They reported 100% sensitivity and 100% specificity of digoxigenin-labelled whole chromosomal DNA probes for 12 of the 14 probes they constructed. Weak cross-reactions were reported between the *S. sanguis II* probe and two strains of *S. mitis*, whereas the *F. nucleatum* probe detected only seven of the 28 strains of *F. nucleatum* used. Therefore only those species previously validated by Gunaratnam *et al* (1992) were considered for use in this study and it was the intention to follow the protocol of these authors precisely to avoid potential cross-reactions and achieve the same levels of sensitivity and specificity. Furthermore, the use of nylon colony lifts taken from primary isolation plates produced results comparable to those achieved using PCM. This was considered important, as culture studies have identified the lists of currently suspected periodontal pathogens and beneficial species. By using the colony lift technique, the final results of this study could then be compared to other centres using DNA probes as well as previous culture studies.

The use of the steroid hapten digoxigenin as a reporter molecule to label whole chromosomal DNA had several advantages over other nucleic acid labels available. The DNA labelling procedure with digoxigenin is relatively simple and requires an overnight reaction. Using this technique, 1 μg of template DNA produces approximately 1 μg of labelled DNA probe, with a molecule of digoxigenin incorporated every 20 - 25 nucleotides. However, the reaction volumes can be scaled up, so that an overnight labelling reaction using 1 μg of template DNA produces 5 - 6 μg of digoxigenin-labelled DNA probe (see below). Digoxigenin is non-isotopic and therefore is less hazardous and easier to handle than radioisotopic labels. Furthermore, digoxigenin-labelled probes are stable for up to one year when stored at - 20°C and can be reused several times. The probes used in this study were used up to nine times with consistent results. Following hybridisation, digoxigenin-labelled probes are detected with anti-digoxigenin antibody conjugated to alkaline phosphatase. The activity of alkaline phosphatase can in turn be detected by a variety of methods, for example NBT/BCIP, naphthol-AS phosphate and diazonium salt colour substrates or chemiluminesent substrate (AMPPD). Usually detection is complete within eighteen hours which is considerably faster than isotopic probes (Smith *et al*, 1989a). By using methods of detection that produce a visible precipitate, the reaction can be stopped at any time once the signals have reached a satisfactory intensity. In the present study the detection of alkaline phosphatase enzyme activity by NBT/BCIP or colour substrates was normally completed within one to three hours.

4.1.2 Modifications

4.1.2.1 Extraction of whole chromosomal DNA

The first method used to extract whole chromosomal DNA from subgingival bacteria was found to be time consuming and although producing DNA of high purity, the total

yield was generally disappointing. Modifications to this method (Dr. Sigmund Socransky, personal communication) provided a faster extraction protocol that improved the yields of whole chromosomal DNA from Gram-negative species. The main differences between these methods were that the modified method achieved cell lysis using sarcosyl rather than SDS and proteinase K as in the earlier method. The modified method also used RNase earlier which allowed a single phenol/chloroform/isoamyl alcohol purification and precipitation of the DNA, immediately after CTAB/NaCl removal of cellular proteins and polysaccharides. The modified DNA extraction protocol used with Gram-positive species required the addition of achromopeptidase with lysozyme and a 10 min incubation at 75°C following the addition of 2% sarcosyl before the addition of RNase. These steps were aimed at improved lysis of the Gram-positive cell wall (Materials and Methods). These amendments generally resulted in increases in yield of DNA from a selection of Gram-postive species. Protein assays, absorbance spectrophotometer measurements and AGE indicated that the DNA extracted using this method was free from contamination of proteins, RNA or phenol (Table 5, Figure 3).

4.1.2.2 Labelling with digoxigenin

Agarose gel electrophoresis of extracted whole chromosomal DNA showed that the DNA had been fragmented into a range of sizes but that the majority was 23.1 kb or larger (Smith *et al*, 1989b). Although whole chromosomal DNA of 23.1 kb could be used successfully labelled with digoxigenin (Boerhinger Mannheim, personal communication) the optimum size of the DNA template for labelling with the random priming technique was in the region of 1 - 2 kb. Shearing whole chromosomal DNA by passing it through a 25 gauge needle four times, reduced the average size of the DNA fragments to between 0.13 and 2.3 kb (Figure 3). The digoxigenin-labelled DNA probes produced from this size of DNA template were also in this region (Figure 4).

In order to produce the quantities of DNA probe required to hybridise colony lifts prepared from patient samples the reaction volume of the labelling reaction was scaled up. Normally, 1 μg of template or unlabelled DNA will produce 1 μg of digoxigenin-labelled DNA if the reaction is allowed to proceed for 20 hours. However, if the reaction volumes are scaled up an overnight labelling reaction using 1 μg of template DNA produces 5 - 6 μg of digoxigenin-labelled DNA probe (Materials and Methods, Boerhinger Mannheim, 1993). This was considered an advantage given the amount and concentration of labelled DNA required to deal with a large number of plaque samples. The original 1 μg of template DNA is not itself labelled with digoxigenin during the procedure therefore the final product of a labelling reaction contains both labelled and unlabelled DNA. The ratio of labelled to unlabelled DNA may affect the strength of the final signal in the following ways. Unlabelled DNA would be expected to compete with labelled DNA for hybridisation with target sequences fixed to the nylon support, hybridise with labelled DNA in solution and be incorporated into amplification trees with a resultant reduction in the concentration of digoxigenin at the hybridisation site and therefore a reduction in the potential signal. If several normal volume reactions were used to generate the amount of labelled DNA probe required then the ratio of labelled to unlabelled would always be 1 : 1. However, the combined products of several scaled up reactions would give a ratio of labelled to unlabelled DNA in the region of 5 or 6 : 1. In the event, no difference between the probes constructed by either method was observed, but the scaled up labelling reactions were used thereafter to produce large quantities of labelled DNA required.

4.1.2.3 Colony lift protocols

Initially, the colony lift protocol as described by Gunaratnam *et al* (1992) was used for both Gram-positive and Gram-negative species. However, there was concern that in the present study this protocol did not consistently lyse colonies of Gram-positive species.

Attempts were made to increase the effectiveness of the lysing stages and involved the use of achromopeptidase and lysozyme (Materials and Methods). These additional stages did result in an improvement in the lysis of Gram-positive species in general. Unfortunately, the amendments to the colony lift protocol were not compatible with Gram-negative species on mixed colony lifts. These colonies were readily dislodged from the surface of the nylon membrane during transfer of the lift from one solution to the next. In this respect, the original colony lift protocol was superior for Gram-negative species as these colonies were normally adherent to the membrane surface. It was decided that, because lysis of Gram-positive bacteria could not be consistently achieved using the original colony lift protocol and that the extended protocol was not compatible with the Gram-negative bacteria, *S sanguis* should be removed from the study.

Following lysis, removal of cellular debris and purification, initially the DNA was fixed to the surface of the colony lift by baking for two hours at 80°C. However, DNA can be covalently bound to the surface of a nylon filter by the reaction of primary amino groups on the nylon surface with DNA thymidine residues on exposure to 254 nm ultraviolet radition for one minute (Coen, 1989; Boerhinger Mannheim, 1993c). This more rapid method was adopted since it did not affect the signals achieved at the detection stage.

4.1.2.4 Dispersal of plaque samples and RTF

Gunaratnam *et al* (1992) achieved dispersal of the subgingival plaque sample with a Kontes sonicating tip. However, some of the subgingival plaque samples in the present study would originate from known HIV seropositive patients and a potentially hazardous aerosol could be produced if a sonicating tip were used, therefore it was decided to use an alternative method of dispersal. Weiner *et al* (1979) proposed that an

ultrasonic waterbath could be used to disperse plaque samples. This has the advantage of achieving dispersal more gently, thereby preserving the viability of organisms that may be susceptible to the effects of sonication by a tip. This method also allowed dispersal within a closed vessel and therefore removed the potential risk of a harmful aerosol. However,the use of an ultrasonic waterbath has some drawbacks. The amplitude of vibration of a sonicating tip can be adjusted and therefore some control over the amount of energy transmitted to the sample via the sonicating tip can be exercised. The sonic energy produced by the fixed piezoelectric transducers of an ultrasonic waterbath will vary depending on the position of the sample vessel in the bath. An attempt to overcome this difficulty was made by observing the effects on small silicon beads in a closed bijou placed at different positions in the ultrasonic bath. Five positions in the bath were chosen that agitated the silicon beads to a similar degree and a frame was constructed to hold the 5 ml of RTF in the sample bijoux just below the surface of the water at these positions. This was used throughout the study to achieve consistency in the dispersal of plaque samples. Further assessment of this method of dispersal was made during the sonication experiment. The aims of this experiment were to assess the effects of sonication using an ultrasonic waterbath on pure cultures of the species under investigation, and to provide a supply of agar plates that contained pure cultures of these species for colony lifting to be used as positive and negative controls in hybridisation experiments. The effects of sonication on bacteria in enclosed bijoux were assessed using dilutions in RTF of pure broth cultures of the selected species. It was felt that the effects of sonication on pure cultures of relatively unaggregated bacteria would be of interest because the CFU on the agar plates would most likely have originated from the free-floating bacteria in the sample solution rather than from the mass of plaque. Therefore the viability and vulnerability of freely-floating bacteria to sonication, away from the protection offered by an aggregated mass of plaque, would determine the bacterial growth on agar plates of dilutions of that sample. This experiment and later observations from subgingival plaque samples, established that this

method of plaque dispersal could be used as an alternative to a sonicating tip in an open vessel without compromising bacterial viability or failing to disaggregate subgingival plaque. Furthermore, a large number of samples could be dispersed at a time, thereby reducing the time between sample collection and plating.

4.1.2.5 Selection of species for enumeration

Prior to analysis of any plaque samples, it was necessary to demonstrate the ability of whole chromosomal digoxigenin-labelled DNA probes to detect target DNA of the selected species on colony lifts. As mentioned above, it proved difficult to achieve consistent lysis of *S. sanguis* using the colony lift protocol that was to be used for subgingival plaque samples and it was decided to leave this organism out of the analysis. However, control hybridisations involving *F. nucleatum* gave inconsistent results although the colony lift was not thought to be at fault. There may be several explanations why *F. nucleatum* proved difficult to hybridise. One may be that the %G + C content of *Fusobacteria* is low being in the region of 27 - 28% which is lower than the other species hybridised, ranging from 38 - 48% (figures from Bergey's Manual of Systematic Bacteriology, 1984). As a result the ideal hybridisation temperature may be lower than the 42°C used with the colony lifts. However %G + C content is believed to have little effect on filter hybridisation in practice (Anderson and Young, 1985). Another reason could be that the spectrophotometer reading of DNA extracted from *F nucleatum* may have overestimated the total yield and have resulted in less than 1 µg of DNA being added to the labelling reaction. In retrospect, examination of the AGE of sheared DNA (Figure 3), the *F. nucleatum* signal appears weaker than the others, although 1 µg of DNA from each of the organisms was added. This could have resulted in the *F. nucleatum* probes being weaker than the others. However, this problem would be expected to manifest itself as the *F. nucleatum* probes becoming exhausted after relatively few hybridisation reactions. In contrast, probes prepared to

F. nucleatum were seen to work inconsistently throughout a series of hybridisation reactions. Previous difficulties in hybridisation with this species have been reported (Gunaratnam *et al*, 1992; Dr. Gillian Smith, personal communication). These problems set against a background of taxonomic difficulties with *Fusobacteria*, (Dzink *et al*, 1990) led to the decision to drop this species from the microbiology assessment. No problems were experienced with the remaining seven species in hybridisation and detection procedures.

4.1.2.6 Clinical samples

At baseline examination, subgingival plaque samples were taken only from the mesiobuccal site of all teeth present, with the exception of third molars, by Gracey curettes. The reason for restricting samples to the mesiobuccal site at this visit was essentially to keep the number of samples to be processed for each patient down to a manageable level. The mesiobuccal site was considered the easiest to sample and would provide one subgingival plaque sample per tooth. This would provide 28 samples from a patient with a full complement of teeth and it was felt that this would allow a reasonable estimate of the mean percentages of probe species for each patient. The choice of Gracey curettes to sample subgingival plaque rather than paper points was in order to keep conditions as close to the Gunaratnam study as possible.

4.1.2.7 Transport and dilution of plaque samples

RTF was chosen as the transport and dilution medium for the plaque samples as it was easier to prepare than pre-reduced anaerobically sterilised modified Ringers solution used by Gunaratnam *et al* (1992). The time from collection of the last sample to placing TSBA plates, containing dispersed and diluted plaque samples, into the anaerobic chamber was generally one hour. This was to ensure that loss of viability of species

sensitive to aerobic conditions was kept to a minimum. The suitability of RTF for these species had already been demonstrated during the sonication experiments when all dilutions were completed in RTF. Finally, dilutions of subgingival plaque samples were completed under a continuous flow of oxygen-free nitrogen again to ensure minimum loss of oxygen-sensitive species, as during the sonication experiment.

4.1.2.8 Prehybridisation and hybridisation of plaque samples

Although the prehybridisation and hybridisation conditions were maintained as described by Gunaratnam *et al* (1992), adjustments were made to the overall procedure by these authors, to ensure improved efficiency in terms of time, cost and labour. Gunaratnam *et al* (1992) used three colony lifts per subgingival plaque sample, cut two of these lifts into fifths and the remaining lift into quarters and hybridised each section with one of their fourteen probes. Successful hybridisation of target DNA with the probe used was detected with anti-digoxigenin antibody and NBT/BCIP. Brown colony forms were enumerated on each of the sections and then these were discarded. However, in this study it was decided to use only one nylon colony lift per site to help keep costs down. In addition, the extra time and labour required to plate, count and colony lift from duplicate or triplicate TSBA plates of each site sampled would have further stretched resources. As a consequence, it was decided to limit the number of species enumerated from each plaque sample to those given in Materials and Methods. However simply cutting each colony lift into eight sections to accommodate these species would have reduced the area of colony lift available for hybridisation with each probe. It was thought this could lead to potential sources of error as it is unlikely that one-eighth of a colony lift will be representative of the growth of the whole agar plate. In order to hybridise a representative section of a colony lift it was necessary to hybridise each section with more than one DNA probe. To analyse the growth produced from each plaque sample with all eight probes, each colony lift was cut into

quarters and each section hybridised with two probes in a sequential manner. Reprobing sections in this way increased the amount of data recovered from each lift while still allowing a reasonable area of each colony lift to be hybridised with each probe.

4.1.2.9 Reprobing membranes

Initially, when NBT/BCIP detection of alkaline phosphatase was used, sections of colony lift were first hybridised, detected and stripped before being reprobed. The reprobing protocol for NBT/BCIP detection involved firstly the removal of the brown precipitate, followed by the removal of the digoxigenin-labelled DNA probe (see Materials and Methods). This method required the use of heated DMF, which is toxic, and did not result in complete colour removal (Figure 9). After stripping the section was then prehybridised, hybridised with another probe, detected and enumerated as before. Although the new coloured precipitate could be easily identified from the blue-ghost colony forms from the previous detection, this method was not ideal and an alternative was sought. Alkaline phosphatase activity can also be detected by production of various azo dyes from the reaction of naphthol-AS phosphates with diazonium salts in the presence of the enzyme (West *et al*, 1990). There are a number of advantages of the use of naphthol-AS phosphates and diazonium salts over NBT/BCIP to detect alkaline phosphatase. These compounds are considerably cheaper, have fast colour development (approximately 90 mins), have very low background, are light-stable and there is a range of colours available (Figure 5). However for detection of bacterial DNA on colony lifts red and blue colour substrates were chosen as they are easily distinguishable at low concentrations. Using these compounds sections of colony lifts could be prehybridised, hybridised with the first probe and detected with red colour substrate solution, then almost immediately hybridised with the second probe and detected with blue colour substrate solution with no stripping procedure with DMF, or second prehybridisation required (Figure 10). On test hybridisations and control colony

lifts no difference in the sensitivity between the two detection methods was observed. Therefore, although both methods allowed the hybridisation of a given area of membrane with more than one probe, the use of colour substrates was preferred as detections were faster and more cost effective.

4.1.2.10 Simultaneous hybridisations

A provisional protocol for simultaneous hybridisation and colour detection was obtained from Boeringher Mannheim, although the colour detection substrates were not yet available from the company. This protocol was adapted for the detection of three subgingival species on nylon colony lifts using the colour substrates mentioned above. Simultaneous hybridisation and subsequent multicolour detection represent another way in which more information can be obtained from a limited area of colony lift. Although the results of this small study are encouraging, many more lifts would have to be hybridised to demonstrate the reproducibility and validity of this technique. While simultaneous hybridisations may not be ideally suited to the enumeration of a range of periodontal species in large number of plaque samples, it may be of benefit in other areas of diagnostic microbiology where there is interest in only two or three species (Tables 6 and 7, Figures 11 a,b).

In summary, a number of modifications to the procedure described by Gunaratnam *et al* (1992) were adopted to fulfil the first objective. These modifications enabled the enumeration of a selection of periodontal bacteria in a large number of subgingival plaque samples more efficiently than previously described, without loss of sensitivity or specificity. Furthermore, these protocols would allow a relatively large number of plaque samples to be enumerated for each patient and an estimate of the prevalence of each probe species within the patient groups under study.

4.1.3 Study Design and Data Generated

Before considering the remaining objectives the design of the study and the data generated will be considered.

4.1.3.1 Patient groups

Twenty-nine HIV seropositive and 27 HIV seronegative patients were included in this study. Both groups had a similar mean age, although the HIV seronegative group included more female members than the HIV seropositive group. Both HIV seropositive and control groups had a similar number of teeth at baseline examination, the total number of sites examined in HIV seropositive patients at this visit was 4500, compared to a total of 4068 sites examined at baseline for HIV seronegative patients.

It could not be claimed that either of these two groups were the result of random sampling. The HIV seropositive patients were either referred from the Royal Infirmary of Edinburgh or the City Hospital of Edinburgh to the Edinburgh Dental Hospital for treatment, or observation of specific oral complaints. A number were self-referred and attended the Edinburgh Dental Hospital as they were unable to find a GDP to carry out routine dental treatment because of their HIV seropositivity. While some of these patients had oral signs of HIV infection at baseline examination, the majority did not (Table 10).

The 27 HIV seronegative patients responded to a poster displayed in the EDH and therefore were limited to either the patients or technical/ administrative staff of the hospital. In the majority of cases these patients were assumed HIV negative, however as mentioned above, two male homosexuals were included in this group as they had recently tested negative for HIV at the time of baseline examination. Patient volunteers

may have been receiving treatment at any department within the hospital, although patients currently receiving treatment in the Periodontology Department, together with patients who had received a course of antibiotics within the previous six months, were excluded.

4.1.3.2 Longitudinal data

This part of the study was essentially a cohort study of a group of HIV seropositive patients who were to be monitored at regular intervals. Unfortunately recruitment of HIV seropositive patients into the study proved difficult. The majority of HIV seropositive subjects in the Edinburgh area acquired HIV as a result of IVDA. When approached, these patients were generally not interested in taking part in such a study. The IVDA that were recruited were poor attenders at recall visits, whereas a number of the homosexual men returned on several occasions. This potential bias should be remembered when considering the conclusions drawn from HIV seropositive patients on a longitudinal basis.

4.1.3.3 Clinical data

4.1.3.3.1 Measurement of attachment loss and calibration study

Measurement of attachment loss by conventional probe was considered to be an adequate means of detecting attachment level changes of the magnitude expected in HIV seropositive patients. Originally, it was assumed that the clinical appearance of HIV-associated periodontal disease, i.e. necrosis, sequestration, linear gingivitis accompanied by attachment loss of more than 3 mm would prompt sampling for microbiology. Cross-sectional measurements had established inter- and intra-examiner correlations and the likelihood of false positives (Table 3). However, longitudinal

observations began to suggest the occurrence of subtle changes in HIV seropositive patients i.e. "conventional" periodontal disease. Therefore a retrospective analysis of data, collected from patients seen on a number of occasions, was undertaken to assess measurement reproducibility on a longitudinal basis and the incidence of 3 mm attachment loss (Table 4). The use of conventional probes in determining attachment level loss on a longitudinal basis has been criticised as having a high false-positive rate (Cohen and Ralls, 1988; Best *et al*, 1990). The false positive rate and level of agreement between replicate measurements calculated from the cross-sectional data in this study agreed with rates reported by Best *et al* (1990, Table 3). Therefore there is a possibility that those sites designated "recently active" in the present study will contain some false-positives due to measurement error. However, reviewing the data collected, only a minority of sites fulfilled the sampling criteria and considerable loss of attachment has been rare in this cohort (Table 35). This is perhaps not surprising given the small number of patients taking part and the prospective cohort design of the study. For these reasons the data collected so far, on a longitudinal basis, are insufficient to determine the rate of periodontal breakdown in the HIV seropositive population at large. However, the use of mean attachment loss as a summary statistic, calculated from single measures of attachment loss by conventional probe, will tend to negate any measurement error at individual sites and is a reliable indicator of the degree of attachment loss suffered by the individual. The allocation of a patient to one examiner for the duration of the study and the calibration of both examiners helped to ensure that measurement of attachment loss was as consistent as possible within the design of the study.

4.1.3.3.2 Dichotomous clinical indicators

The choice of simple dichotomous clinical indicators of periodontal disease, rather than other semi-quantitative indices available, had the advantages of good intra- and inter-

examiner correlation without the need for lengthy calibration of these measures as well as reducing the time required for full mouth examination. However, the lack of any quantitative information with regard to the clinical indicators chosen limits speculation on the degree of influence of these factors on each other and their relationship with periodontal disease in HIV seropositive subjects.

4.1.3.4 Microbiological data

Microbiological data were not available for all the sites sampled for a number of reasons. One of the main considerations was to keep the lower limit of detection as low as possible, while still being able to obtain a reasonable estimate of the total colony count for each plate. Sites were only included in this analysis if they had between 100 and 2000 colonies on the primary isolation plate. However only one colony lift from one dilution per site sampled was feasible, therefore if a subgingival plaque sample was either very big or very small, the total colony count of the primary isolation plates did not fall between the above limits and sites were lost due to over- or underdilution of plaque samples. Other common causes of lost samples included drying out of thinly prepared agar plates or contamination of plates with fungi. Although with experience the number of sites that were lost due to these causes reduced, under- or overdilution of plaque samples continued to be a problem throughout the study. It was decided therefore that only those patients with microbiology results from at least 10% of all sites would be used in the statistical analyses. This resulted in 43 patients from a total of 56 patients in the study. Nine patients were excluded by the 10% rule, the colony lifts from the other four patients were lost due to equipment failure during a hybridisation experiment.

4.1.3.5 Immunological data

Immunological data could be traced for most patients with the assistance of the HIV Reference Laboratory in Edinburgh. Two patients provided incorrect dates of birth and therefore their immunological results could not be traced. The criteria set out in the Results section for the inclusion of patients in the statistical analyses excluded a further five patients because more than three months had elapsed between immunological results and baseline examination for these patients. Statistical analysis was restricted to immunological data of 22 HIV seropositive patients. The timing of immunological tests often did not coincide with the baseline periodontal examination examination and this should be considered when comparing immunological results against other parameters, given the labile nature of the $CD4^+$ T-cell counts in particular.

4.1.4 Interpretation of Results

The second and third objectives were to compare the prevalence of periodontal disease and the prevalence of the selected periodontal bacteria between the HIV seropositive and HIV seronegative subjects recruited into the study. Initial analysis of baseline clinical and microbiological data centred on comparisons between the HIV seropositive patients and the HIV seronegative controls. When mean attachment loss of each subject in these two groups was plotted against age, it was immediately apparent that a number of HIV seropositive subjects had increased mean attachment loss for their age compared to the HIV seronegative controls (Figure 16). However, when a Mann-Whitney U test was used to compare mean attachment loss between these two groups the HIV seropositive patients were found only to have a slight tendency for a higher mean attachment loss compared to the HIV seronegative patients (Table 14). Plotting the location of sites with attachment loss of 3 mm or more revealed that the HIV seropositive patients in this sample had more sites with this degree of attachment loss

than HIV seronegative subjects (Figures 17 and 18). Furthermore, the pattern of attachment loss for both groups was different, with the lower incisors of the HIV seropositive subjects experiencing severe attachment loss compared to the same region in HIV seronegative subjects. Comparison of the prevalence of dichotomous clinical indicators revealed that the only difference between these two groups at baseline examination was that the HIV seropositive group had a higher mean percentage of sites exhibiting suppuration than the HIV seronegative group (Table 19). However, this was only a very small difference the median mean percentage of sites with suppuration in HIV seropositive subjects was only 2.6%. Finally, the microbiological results revealed that the only difference between these groups was that the HIV seropositive patients had a statistically significantly higher mean percentage of *P. gingivalis* than the HIV seronegative patients. Therefore, there were only minor differences between HIV seropositive and HIV seronegative groups with respect to prevalence and pattern of periodontal disease and periodontal species enumerated in subgingival plaque.

Using criteria outlined by Haffajee and coworkers (1992) the incidence of widespread and localised periodontal disease was assessed in both groups. Whereas nine HIV seropositive patients fell into the widespread disease category, only two of the HIV seronegative patients were considered to have widespread disease. In the absence of figures for the prevalence of widespread periodontal disease in the general population, these two groups were compared using a chi-square test (Table 16). This showed that this difference in periodontal disease pattern between these two groups was very close to statistical significance ($p = 0.059$). When these two groups of HIV seropositive patients were compared, perhaps not surprisingly, the nine widespread periodontal disease patients had a statistically significantly higher mean attachment loss than the remaining 20 HIV seropositive localised periodontal disease patients ($p = 0.001$, Table 16). When the location of sites with attachment loss of 3 mm or more was compared in HIV seropositive subjects with widespread and localised periodontal disease, it was

evident that the distinct pattern of attachment loss observed in the HIV seropositive patients as a group, was due almost entirely to these nine widespread periodontal disease patients, which included the pattern of attachment loss associated with the lower incisors of these patients (Figures 18, 19 and 20). Furthermore, these nine HIV seropositive widespread periodontal disease patients were also found to have a statistically significantly higher mean percentage of sites with redness and suppuration than the localised periodontal disease patients in this group ($p = 0.030$, $p = 0.001$; Table 20). Further evidence for the existence of a distinct subgroup of HIV seropositive patients came from the microbiology results at baseline examination. Comparison of these two groups revealed that the seven probe species accounted for a higher mean percentage of the cultivable microbiota in the widespread disease patients than in the localised disease patients. Furthermore, these seven HIV seropositive patients had a tendency for higher mean levels of *P. gingivalis, P. intermedia, C. ochracea* and *V. parvula* than the HIV seropositive localised disease patients (Table 29).

Interestingly, when the localised periodontal disease patients from HIV seropositive and HIV seronegative groups were compared, no differences of statistical significance could be found with respect to mean attachment loss (Table 18). Similarly, no differences were found between these two groups when prevalence of dichotomous clinical indicators was compared (Table 21). These two groups were also found to have a similar pattern of attachment loss when sites of 3 mm or more were charted across the mouth (Figures 17 and 20). Finally, no differences of statistical sigificance could be found between localised periodontal disease subjects of both groups in the prevalence of any of the probe species or total percentage of cultivable microbiota identified (Table 30).

Collectively, these results suggest that the HIV seropositive subjects in this sample consisted of two well defined subgroups. One subgroup had widespread periodontal

disease and differences were observed in the prevalence and pattern of periodontal disease, clinical signs of periodontal disease and subgingival microbiota in terms of those species enumerated, compared to the remaining HIV seropositive subjects in this sample with localised periodontal disease. Furthermore, the HIV seropositive subjects with localised periodontal disease were not dissimilar to the HIV seronegative localised periodontal disease group with respect to these parameters. Therefore, the initial differences of mean attachment loss, percentage of sites with suppuration, location of attachment loss and mean percentage of *P. gingivalis* observed between HIV seropositive and HIV seronegative groups could all be attributed to these nine HIV seropositive patients with widespread periodontal disease. No common factor could be found with respect to age, sex, suspected route of acquisition of the virus, time since diagnosis of HIV seropositivity, CDC stage of HIV disease, $CD4^+$, $CD8^+$ T-cell counts or ratio, medication related or unrelated to treatment of HIV infection, that correlated with the existence of widespread periodontal disease in these subjects. Why the lower incisor region of this group had experienced severe attachment loss is also not known. In reviewing the literature, however a number of case reports of HIV-associated periodontal disease have described rapid attachment loss localised to the anterior teeth (Andriolo *et al*, 1986; Abel and Andriolo, 1989; Levine and Glick, 1991). However, only three of the HIV seropositive patients in this study with widespread periodontal disease give a history of previous periodontal problems involving the lower anteriors. The majority of widespread periodontal disease patients had no history of aggressive periodontal destruction, i.e. HIV-P, necrotising stomatitis, therefore it is likely that attachment loss in this group of patients proceeded insidiously, albeit at a faster rate than others of a similar age.

In retrospect, the existence of possible subgroups within the HIV seropositive patients was indicated earlier in the analysis of CTENP as a summary statistic for attachment loss (Figures 12 and 13). Whereas the transformation $\log_{10}$ (CTENP + 1) for HIV

seronegative subjects produced a normal distribution, the same transformation failed in the HIV seropositive subjects; the distribution produced was not normal and had at least two distinct peaks. This appearence is often an indication that subgroups are contained within a sample.

Reports of the prevalence for selected periodontal bacteria vary greatly for both HIV seropositive and HIV seronegative patients at a site and subject level (Dahlén *et al*, 1989; Zambon *et al*, 1990; Savit and Kent, 1991; Asikaninen *et al*, 1991; Moore *et al*, 1984, 1985, 1991; Moore *et al*, 1993). Prevalence will depend on number of sites sampled per patient, method of bacterial identification and detection limits (Mombelli *et al*, 1991a,b). Tables 26 and 27 give the prevalence of the species enumerated at a subject and site level from both groups. While the majority of these results for prevalence of the seven probe species are comparable to results of prevalence from the studies by Gunaratnam *et al* (1992) and Haffajee *et al* (1992), the prevalence of some species in this study population were different. In the present study, *A. actinomycetemcomitans* was found at a higher prevalence at a subject and a site level compared to these studies, whereas the prevalence of *P gingivalis* in sites from HIV seropositive was found to be lower than that reported by Gunaratnam *et al* (1992). However, it should be remembered that although all three studies used the same method of enumeration of subgingival bacteria, there were a number of differences in the clinical and laboratory methods, as well as sample population that could explain these discrepancies. As discussed above, both the transport medium and method of plaque dispersal used in this study were different from that used by the group at Forsyth Dental Center which could lead to differences in the viabilty of organisms following dispersal of a plaque sample. Secondly, the patients in this study were not exclusively periodontitis patients as was the case with the patients in the FDC studies. Furthermore, the Edinburgh population has not been previously analysed with respect to these species and geographic variation in the prevalence of these species should be considered as a

possible cause of differences (Prof. T.W. MacFarlane, personal communication). This concern confirmed the need for a separate HIV seronegative control group in this study. Finally, a priority in the present study was to keep the lower limit of detection as low as possible. This was achieved by reprobing sections of colony lift and lifting primary isolation plates with total colony counts of up to 2000. This resulted in a "best" lower limit of detection of 0.2% of the total colony count and a mean lower limit of detection of 0.58 % for HIV seropositive patients and 0.60% for HIV seronegative controls. This compares to a "best" lower limit of detection of 0.4% for other studies and may account for some of the differences observed between these studies. The detection limit of a technique will have a profound effect on the observed prevalence of organisms at a site and subject level. Results from Savitt and Kent (1991) show a dramatic increase in the percentage of sites positive for both *P. gingivalis* and *A. actinomycetemcomitans*, when the sensitivity of the test was improved from 10^5 to 10^3 organisms. The data reported by these authors show an increase from an average percentage of sites positive for *P. gingivalis* from 14.4 % of sites to 38.9% of sites with the lower detection limit. Similarly, the average percentage of sites positive for *A. actinomycetemcomitans* increased from 3.7% to 41.9% when the detection limit was lowered to 10^3 cells. As discussed above, the increased sensitivity of the rapid identification techniques have called into question the dependence on culture techniques as the gold standard for bacterial identification in subgingival plaque samples. As a consequence, currently suspected periodontal pathogens either may be more prevalent than once thought or the list of currently suspected periodontal pathogens and beneficial species may be inaccurate (Smith, 1992).

In this study, no correlation could be found between mean attachment loss or mean percentage of the seven probe species and $CD4^+$, $CD8^+$ T-cell counts or ratio. No differences were found with respect to dichotomous clinical indicators and $CD4^+$, $CD8^+$ T-cell counts or ratio, with the possible exception of percentage of sites with

redness and $CD4^+$: $CD8^+$ ratio. A weak negative correlation was found between $CD4^+$: $CD8^+$ ratio and percentage of sites with redness, however this was not statistically significant (Figure 21). The lack of correlation found between immunological results and the clinical indicators and microbiological results could be the result of the variable time lapse between immunological results and baseline examination. Furthermore, the the lack of quantitative information provided by dichotomous measures could result in a failure to detect an association between more subtle clinical changes and immunological parameters.

On a longitudinal basis some differences in the clinical parameters studied was observed in the HIV seropositive subjects. Pocket depth was seen to improve in 14 of the 18 HIV seropositive patients at a second visit (Table 22). This was accompanied by a reduction in the percentage of sites with bleeding on probing over the course of the first few months and paradoxically, by an increase in the percentage of sites with plaque. The reduction in pocket depth and bleeding on probing between baseline examination and second visit for some of these patients may be a result of the scaling and oral hygiene instruction given at baseline visit and an increased awareness that periodontal problems could occur as a result of HIV infection. However, at the end of three months patients may have lapsed back into previous standards of plaque control resulting in higher plaque scores (Tables 22 and 23).

The incidence of periodontal breakdown of at least 3 mm in HIV seropositive patients seen at recall visits was extremely rare (Table 35). Only 20 sites from a total of 2814 reviewed on at least one occasion were deemed active, which gives an average rate of breakdown across visits 2, 3 and 4 of less than 1.0% (Table 35). Interestingly, this is well below the false-positive rate predicted for this method of measuring attachment loss (Best *et al*, 1990). These authors predicted that using this method of measurement at least one site in every patient would record a change of 3 mm at every follow up visit

due to measurement error alone. Perhaps using only two calibrated examiners in this study helped keep the false-positive rate down. If the criteria for active sites had been 2 mm or more of attachment loss then number of active sites detected in this study would no doubt have been larger, but at the same time the false-positive rate would also be expected to rise. Although some of these sites had the clinical appearance of bleeding on probing, redness and suppuration, none of the sites designated active came from areas of HIV -G, HIV -P or NS. The incidence of these diseases in this sample of HIV seropositive patients was restricted to three patients at baseline examination (see below). There were no differences of statistical significance found between active and control sites from HIV seropositive patients with regard to clinical indicators or the species enumerated. This is perhaps not surprising given the numbers of sites available for use in the statistical analysis, and as a consequence, the power of these tests would be expected to be low.

The next two objectives outlined were to find the prevalence of HIV-associated periodontal diseases in a sample of HIV seropositive subjects and attempt to characterise the microbiology of these lesions in the context of those species selected for enumeration. However the incidence of these conditions in this sample of HIV seropositive subjects during the study was extremely low and, as discussed above (section 4.1.3.2), the number of HIV seropositive patients recruited and the prospective design of the study may have been contributory factors. Of 29 HIV seropositive patients, nine had a history of periodontal treatment, however with the exception of one patient, an accurate dental history was not available and so it was impossible to classify previous disease experience in terms of the conditions listed above. Furthermore, although many of these patients had clinical evidence of periodontal pocketing and localised areas of gingival recession, it was impossible to determine if periodontal destruction had occurred before or after HIV infection. One patient had a previous episode of HIV -P that progressed to necrotising stomatitis and resulted in an oro-antral

fistula (Felix *et al*, 1991). At baseline examination, only 2 patients with a previous history of periodontal disease presented with clinical signs. One patient had suspected ANUG around the lower incisors, the other had generalised HIV -P with localised areas of HIV -G. One patient, with no previous history of periodontal disease, presented at baseline examination with suspected ANUG also localised to the lower incisor region. On a longitudinal basis, 7 patients had attachment loss of 3 mm or more between subsequent visits in 20 sites from 2814 sites reviewed on at least one occassion (see above). None of these cases of active disease were attributed to HIV-associated diseases as described in the literature. It is possible that three monthly recall visits with oral hygiene instruction and scaling, if required, may have reduced the incidence of severe HIV-associated periodontal disease in this group.

Finally, it was hoped to identify any possible predictors of HIV disease progression in terms of the clinical and microbiological parameters under study. Ideally, this aim would have been satisfied by statistical analysis of longitudinal data. Due to difficulties in recruitment of patients and patient drop-out, the majority of the data collected were cross-sectional. In an attempt to provide some information on possible periodontal indicators of HIV disease progression, cross-sectional data was used with a Kruskal-Wallis test across the three patient groups that crudely divided patients into stages of disease progression, i.e. HIV seronegative, CDC stage II and CDC stage IV. The results of these analyses should be viewed with caution, as, ultimately, the conclusions drawn originate from cross-sectional data. Longitudinal studies will be required to verify any of these findings.

Mean attachment loss and the percentage of sites exhibiting suppuration were greater in CDC stage IV subjects than either CDC stage II or HIV seronegative patients (Tables 41 and 42). Interestingly, CDC stage II patients had a similar degree of attachment loss to the HIV seronegative subjects, whereas CDC stage IV patients had

on average a higher mean attachment loss. On the basis of these data, CDC IV subjects would appear to have suffered either more episodes of active periodontal destruction or episodes of increased severity than either CDC II or HIV seronegative subjects of a similar age. It is tempting to speculate that increased periodontal activity occurs at or around the time of increased immunosuppression associated with the progression of a patient from CDC stage II to CDC stage IV. HIV disease progression from CDC stage II to CDC stage IV is associated with increased immunosuppression which in turn leads to increased susceptibility to opportunistic pathogens. This process may also be presumed to occur in relation to subgingival bacteria in some patients. Indeed increasing immunosuppression may explain the differences noted in the mean percentage of some of the species enumerated across the three groups. The mean percentage of *P. gingivalis* was observed to increase across the groups with the highest mean percentages occuring in CDC stage IV subjects. The mean percentages of *V. parvula* were highest in the CDC stage II subjects, but very low in CDC stage IV subjects (Table 43). It may be that these fluctuations of *P. gingivalis* and *V. parvula* confirm their description as a suspected periodontal pathogen and beneficial species respectively. However, even if these associations are upheld in longitudinal studies, the predictive power of mean attachment loss and levels of *P. gingivalis* or *V. parvula* are likely to be weak, as not all HIV seropositive patients will suffer from periodontal disease or harbour these organisms.

4.2 SPECULATIONS ON PERIODONTAL DISEASE IN HIV SEROPOSITIVE SUBJECTS

Results from PCM, immunofluorescence, ELISA and DNA probe studies of the subgingival microbiota of HIV seropositive patients and HIV-associated periodontal disease have found similar organisms present to those found in healthy sites and

periodontal disease sites in HIV seronegative subjects (Murray *et al*, 1988, 1998, 1991; Zambon *et al*, 1990; Lucht *et al*, 1990; Rams *et al*, 1991; Gornitsky *et al*, 1991; Moore *et al*, 1993). Results from this study also confirm the existence of a selection of currently suspected pathogens and beneficial species in HIV seropositive subjects. However, several studies have also found species that are not normally considered colonisers of the subgingival crevice in HIV seropositive patients (see Literature Review section 1.8.2). Therefore, there are two microbiological hypotheses that could apply to the aetiology of HIV-associated periodontal diseases. Either they could be caused by subgingival species currently associated with conventional periodontitis, but whose virulence is increased with the diminished immune response in HIV infection, or be the result of infection by the unusual species found colonising the subgingival area in HIV seropositive subjects (Robinson 1992). As discussed previously, HIV infection has been reported to cause defects in PMNL, macrophages and B-lymphocyte function as well as $CD4^+$,$CD8^+$ T-lymphocytes and cytokine production (Literature Review, section 1.8). Similar defects in PMNL, macrophages B-and T-lymphocytes have been reported in some patients with various periodontal diseases (Literature Review, section 1.6). Given the demonstration of both suspected periodontal pathogens and unusual colonisers in the subgingival crevice of HIV seropositive subjects, and the many host cell and immune system defects that have been reported in HIV seropositive subjects, it is perhaps not surprising that a percentage of HIV-seropositive patients should suffer from aggressive periodontal disease. The fact that all HIV-seropositive subjects in the present study did not exhibit severe disease may be the result of the small number seen longitudinally, individual variation or the effects of various medications.

Periodontal destruction could occur in HIV seropositive patients in a number of ways. Conventional periodontitis and gingivitis, in response to fluctuations in the quantity and possibly quality of supragingival and subgingival plaque, will exist in some patients before HIV infection. It seems unlikely that the periodontal disease process will change

in character in the early stages of HIV infection, when the immune system and the resident microbiota are still relatively intact. As HIV disease progresses, patients may be more likely to experience changes and imbalances in the subgingival microbiota due to immunosuppression and treatment of manifestations of HIV infection. As a consequence, they may suffer either increased frequency or severity of active periodontal disease, although the clinical characteristics may still be similar to conventional periodontal disease. Interestingly, depressed $CD4^+$: $CD8^+$ ratio, defects in PMNL and AMLR function as well as B-lymphocyte hyper-responsiveness have been associated with both HIV infection and early onset periodontal disease (see Literature Review). Furthermore, RPP has been described as an early expression of HIV disease (Rosenstein *et al*, 1989; Tenenbaum *et al*, 1991; Levine and Glick, 1991). Finally, in a percentage of HIV seropositive subjects, as immunosuppression increases the subgingival crevice may be free from any effective host influence, allowing both periodontal pathogens to increase and unusual subgingival species to colonise, giving rise to severe periodontal diseases i.e. necrotising stomatitis and HIV-P. It is likely that a number of these conditions coexist at different sites in the same patient.

Although the results of this study indicated a tendency for mean attachment loss to increase across CDC stages, the presence of widespread periodontal disease or the incidence of active sites were not restricted to those patients with increased immunosuppression. It is likely that many other factors will also play a role. Such factors may include presence of risk factors for conventional periodontal disease, smoking, OH status, poor dental health, previous history of conventional periodontal disease and genetic susceptibility to periodontal disease. However, antimicrobials used in the treatment of opportunistic infections in HIV seropositive subjects could have a profound effect on the subgingival microbiota. This could lead to the emergence of opportunistic periodontal pathogens, that in turn may explain the poor response to treatment of HIV-associated periodontal diseases reported by some authors (Winkler

and Murray, 1987; Grassi *et al*, 1988). Indeed, the existence of an established protective subgingival microbiota against opportunistic and exogenous periodontal pathogens prior to and during HIV infection and emergence of opportunistic pathogens in an established subgingival microbiota in the presence of failing immunoregulation are interesting possibilities to consider in this group of patients.

It seems therefore, that the ability to identify HIV seropositive subjects at risk of severe periodontal disease will be as difficult as predicting such disease in non-immunocompromised individuals.

Although the present study was originally designed to collect and analyse both cross-sectional and longitudinal data, for a variety of reasons, the majority of the data collected was cross-sectional. Longitudinal observation of HIV seropositive subjects may offer answers to the following:

1) When does increased periodontal disease activity observed in some HIV seropositive patients take place in relation to stage of HIV disease?

2) Is the increased activity observed in these patients related to an increase in the number or severity of destructive phases of the disease?

3) Why should the lower incisors of these patients be the focus for severe disease?

4) Could fluctuations in specific subgingival species be related to increased immunosuppression?

5) How do HIV seropositive subjects compare with subjects with early onset periodontitis e.g. rapidly progressive periodontitis?

The development of additional naphthol-AS phosphate / diazonium salt colour substrates to detect alkaline phosphatase activity, would allow more data to be recovered from each colony lift if a sequential hybridisation strategy were adopted. This would also allow more simultaneous hybridisations to be attempted as new non-isotopic nucleic acid labels are developed.

4.3 SUMMARY

The results of this study suggest that there exists a subgroup of HIV seropositive patients that are at risk for severe or widespread periodontal disease. Whereas a percentage of HIV seropositive subjects will suffer from severe, aggressive periodontal destruction i.e. HIV-P, necrotising stomatitis, a number of these patients will develop a form of rapidly progressive periodontal disease. One of the features of this disease may be that the lower incisors of some subjects are at risk for attachment loss. HIV disease progression may also be associated with fluctuations in subgingival microbiota i.e. increasing levels of *P. gingivalis* and falling levels of *V. parvula*, but this will require further investigation with a larger number of patients.

No clinical or microbiological features could distinguish HIV seropositive subjects and HIV seronegative subjects with localised periodontal disease. Despite five of these HIV seropositive subjects having full blown AIDS, increased periodontal destruction did not appear to be a feature of their immunosuppression. It may be that other factors would help explain absence of severe periodontal destruction in every case of HIV infection, for example, colonisation resistence offered by an intact subgingival microbiota in disease resistant HIV seropositive patients.

This study has confirmed that DNA probes are a simple and useful technique to enumerate a large number of bacterial species in a large number of samples. Development of the technique used in this study, i.e. sequential hybridisation of target with a number of probes and detection of alkaline phosphatase with naphthol-AS phosphates and diazonium salts, has allowed increased data recovery from a single colony lift without necessarily incurring additional costs. In addition to sequential hybridisation, simultaneous hybridisation with multicolour detection also provides a way of increasing data recovery from a limited area of filter. The choice of technique

will depend on the number of species and the number of samples under study. For a large number of samples and species, sequential hybridisations would seem more appropriate. However, for a small number of both, simultaneous hybridisation may be quicker, allowing colony lifts to be hybridised without requiring to be sectioned.

Over the last five years there has been a great increase in the number of rapid microbial identification techniques available (see Literature Review section 1.5). These techniques, while differing in sensitivity and specificity of detection of the target species, have allowed the analysis of a greater number of plaque samples than was previously possible using a cultural approach. However, questions remain about the ability of a specific microbiota to indicate/predict subject or site-specific periodontal disease activity. The use of these techniques in studies analysing a larger number of sites within subjects than previously, may help in clarifying the lists of currently suspected periodontal pathogens and beneficial species, however controversy over the specific or non-specific plaque hypothesis is unlikely to be completely resolved until periodontal disease activity can be confidently identified using a single visit test. Microbial analysis of plaque samples for selected pathogens or beneficial species from sites known to be active at the time of sampling will make the determination of the contribution of each species to the destructive disease processes much easier.

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APPENDICES

Appendix 1

A BROTHS

1. Bacteroides medium CMB

Cooked meat broth (CMB) modified by the addition of;

trypticase (BBL)	1%
protease peptone (Oxoid)	1%
yeast extract (Difco)	0.5%
sodium chloride	0.5%
sodium succinate	0.25%
cystine hydrochloride	0.075%
hemin	5 mg/ml
menadione	1 mg/ml

2. Bacteroides forsythus

Brain heart infusion broth (Difco) supplemented with;

fetal calf serum	5%
N-acetylmuramic acid	10 mg/ml

3. Campylobacter rectus

Mycoplasma broth supplemented with;

brain heart infusion	37 mg/ml
hemin	5 mg/ml
sodium formate	2mg/ml
sodium fumerate	3 mg/ml
yeast extract	5 mg/ml.

B SOLID MEDIA

1. Porphyromonas gingivalis agar

(TSBA HM)

Trypticase soy agar supplemented with;

horse blood	5%
hemin	5 mg/ml
menadione	1 mg/ml

2. Bacteroides forsythus agar

TSBA base	50%
BHI A base	50%
horse blood	5%
N-acetylmuramic acid	10 mg/ml

2. Campylobacter rectus agar

BHI agar	52 mg/ml
yeast extract	10 mg/ml
sodium formate	2 mg/ml
sodium fumarate	3 mg/ml
potassium nitrate	2 mg/ml
hemin	10 mg/ml

C REAGENTS

1. Reduced Transport Fluid

(Syed and Loesche, 1972)

One litre of RTF consisted of the following;

Stock salt solution No.1	75 ml
Stock salt solution No.2	75 ml
8% $Na_2 CO_3$	5 ml
1% solution of dithiothreitol (freshly prepared)	20 ml
0.1% solution of resazurin	1 ml
distilled water	824 ml

Stock salt solution No. 1 is a 0.6% solution of potassium hydrogen phosphate.

Stock salt solution No. 2 is a mixture of the following;

1.2% sodium chloride
1.2% ammonium sulphate
0.6% potassium dihydrogen phosphate
0.25% magnesium sulphate

2. CTAB/NaCl

10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl

3. RNase A (stock solution)

10 mg/ml RNase A in 10 : 1 TE (pH 7.6) heated to 70°C for 15 min and stored in 100 ml aliquots at -20°C

4. 24: 25: 1 phenol : chloroform: isoamyl alcohol

Buffered Phenol

1) Add 250 ml 50 mM Tris base to 250 ml liquified phenol
2) Stir for 10 mins with magnetic stirrer in covered beaker
3) Let phases settle
4) Decant top phase and discard
5) Remove last traces of top phase with pipette
6) Add 250 ml 50 mM Tris HCL, pH 8.0
7) Repeat steps 2 - 6
8) Check pH of phenol phase with indicator paper (pH 8.0)
9) Add 125 ml TE buffer
10) Store at 4°C in brown glass bottle
11) Mix 25 vol buffered phenol, 24 vol chloroform and 1 vol isoamyl alcohol

5. 20 x SSC

3 M sodium chloride

0.3 M tri-sodium citrate, pH 7.0

6. De-ionised formamide

5g of Dowex MR-3 ion exchange resin added for every 50 ml of formamide. Stirred for 30 mins at room temperature and filtered twice through Whatman No. 1 Filter paper. Dispensed into aliquots and stored at - 20°C.

7. 50 x Denhardt's

1% (w/v) Ficoll
1% (w/v) polyvinylpyrrolodone
1% (w/v) bovine serum albumin, fraction V
in distilled water.

Filter sterilised and stored in 15 ml aliquots at -20°C

8. Herring sperm DNA

One gram of HsDNA dissolved in 100 ml distilled water overnight at 4°C. Sheared by passing through a 25 gauge needle four times, stored in 1 ml aliquots at -20°C.

9. Nitroblue tetrazolium (stock solution)

75 mg/ml nitro blue tetrazolium salt in dimethylformamide (70% v/v).

10. Bromo-chloro-indolyl-phosphate (stock solution)

50 mg/ml of 5-bromo-4-chloro-3-indolyl phoshate, toluidinium salt, in dimethylformamide (100% v/v).

11. Fast red TR (stock solution)

4-Chloro-2-methylbenzene diazonium salt hemi zinc chloride salt, 20 mg/200 ml in dimethyformamide (100% v/v).

12. Fast Blue BB (stock solution)

4-Benzoylamino-2,5-diethoxybenzene diazonium chloride hemi [zinc chloride] salt 20 mg/200 ml in dimethylformamide (70% v/v).

The fast blue BB salt did not dissolve completely in the dimethylformamide and was vortexed and intermittently shaken for 5 min, followed by 5 min centifugation and the supernatant tranfered to a fresh Eppendorf before use.

13. Naphthol-AS phosphate (stock solution)

20 mg/200 ml in dimethylformamide (100% v/v).

14. Naphthol-AS-GR phosphate (stock solution)

20 mg/200 ml in dimethylformamide (100% v/v).

15. Colour Substrate Solutions

All colour substrate solutions allow 50 ml of solution for every 50 cm^2 of filter.

Red

10 ml of naphthol-AS phosphate stock and 3 ml of fast red TR stock in 5 ml of buffer 3.

Blue

10 ml of naphthol-AS phosphate stock and 15 ml of fast blue BB stock in 5 ml of buffer 3.

Green

10 ml of naphthol-AS-GR phosphate stock and 15 ml of fast blue BB stock in 5 ml of buffer 3.

Appendix 2

A) Example of record sheet for HIV seropositive subjects

Patient No. .....

Visit No. .....

Systemic

HSV
HZ
PCP
KS
CMV
Other

Medication

Penicillin
Metronidazole
Pentamidine
Other

Nystatin
Amphoteracin
Fluconazole
Other

Acyclovir
AZT
DDI
Other

Oral

Candida
Herpes
HPV
KS
OHL
ROU
Pigment
ITP
SGE
Perio

CDC

Appendix 2

B) Example of a chart used to record clinical indicators.

URB		N	A	B	A-B	BOP	PLQ	RED	SUP	SEQ
17	3									
	2									
	1									
16	3									
	2									
	1									
15	3									
	2									
	1									
14	3									
	2									
	1									
13	3									
	2									
	1									
12	3									
	2									
	1									
11	3									
	2									
	1									

URB = upper right buccal quadrant
173 = tooth 17 / site 3 = distobuccal
172 / site 2 = buccal
171 / site 1 = mesial

N	=	necrosis	BOP	=	bleeding on probing
A	=	pocket depth	PLQ	=	plaque
B	=	level of ACJ	RED	=	redness
A-B	=	attachment loss	SUPP	=	suppuration
			SEQ	=	sequestration